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Behavioral and physiological responses of a freshwater turtle to anthropogenic activity

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Behavioral and physiological responses of a freshwater turtle to anthropogenic activity

by

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DEDICATION

To my wonderful family, who has always caught wild herpetofauna with me, found this passion endearing, and nurtured it.

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ABSTRACT

The way an animal perceives and reacts behaviorally to its environment is mediated largely by its physiology. For example, in vertebrates, glucocorticoids are released in response to a perceived stressor and help to keep the animal in a heightened state of awareness to enable an appropriate response to the perceived threat. It is also possible for organisms to react in non-adaptive ways to novel stressors. For example, an organism could perceive novel species, such as humans, as threats, when in fact the humans are engaging in recreational activity. This incorrect assessment could lead to wasted energy (particularly detrimental in poor-quality habitats) or the desertion of quality habitat, often to species that react less strongly to the presence of humans. It could potentially even lead to chronic elevation of glucocorticoids, reducing ability to mount a stress response to actual life-threatening stressors. Additionally, chronically elevated glucocorticoid levels may affect the fitness of the offspring of stressed individuals.

These physiological responses can alter fitness sub-lethally. Such sub-lethal effects could reduce population fitness more than lethal impacts because more individuals might experience acute stress that interferes with reproduction. Thus, understanding organismal physiology and how it is modified in response to novel stressors, including habitat loss, human recreational activities, or even invasive species, is a key way to examine individual and population-level reactions to anthropogenic activities.

Using the widespread freshwater turtle, *Chrysemys picta,* as a model system, I identified responses to anthropogenic stressors and consequences of transgenerational transmission of stress effects. The research presented here quantifies these stress responses across several levels of biological organization: measures of multiple traits within individuals as well as repeated measures over time, comparisons of different populations to understand how the stress response

varies across an urban-rural gradient, and degrees of individual differences in physiology and behavior in offspring exposed to varying levels of simulated maternal stress at oviposition to understand the consequences of heightened maternal stress on fitness.

In the first study, I measured an important biomarker of the stress response, plasma corticosterone (CORT), in two populations of *C. picta* that differed primarily in the level of human recreational activity to which they were exposed during the reproductive season. Individuals from both populations exhibited similar levels of circulating CORT despite drastic differences in the number of humans they encountered while performing critical behaviors such as basking, mating, and nesting. In the second study, I measured differences in an important behavioral measure, flight initiation distance, between these same two populations of *C. picta*. Turtles from the population exposed to few humans had longer flight initiation distances than the population exposed to thousands of humans engaged in common recreational activities in the area. That is, turtles less familiar with humans did not allow researchers to approach as closely as did turtles that encountered humans on a regular basis. In the final study, I topically applied CORT to recently laid *C. picta* eggs as a proxy for three levels (low, medium, and high) of maternal stress. I measured important markers of hatchling fitness, such as survivorship to hatch, size, and mass, performance in righting trials (to replicate predation events), and dispersal ability. Elevated levels of CORT at oviposition decreased probability of embryos surviving to hatch. Furthermore, hatchlings from CORT-treated eggs did not right themselves as often as their conspecifics, but were quicker when they did. However, these behavioral differences did not translate into differential survivorship in the release experiment. This body of work provides insight into how freshwater turtles may react to increasingly human-modified environments.

CHAPTER 1

INTRODUCTION

Organisms are exposed to a variety of stressors throughout their lifetimes that pose challenges to reproduction and survival. Stressors can take many forms. They are often divided into predictable recurring stressors and unpredictable life-threatening stressors (Landys et al. 2006). Predictable stressors are associated with a suite of behaviors and physiological adjustments that can change with annual, expected events, such as breeding (O'Reilly and Wingfield 2001) and migration (Ramenofsky and Wingfield 2007). This suite of changing behaviors and physiology in response to recurring environmental change is often defined as allostasis, or the ability to adjust physiology to a new baseline as the season or situation requires (Landys et al. 2006). As one would expect, allostasis facilitates major adjustments in levels of circulating hormones, behavior, and body fat composition, to name just a few. These changes are predictable, and cause physiological and behavior fluctuations that follow similar patterns from year to year.

Alternatively, unpredictable, often life-threatening stressors, or labile perturbation factors (LPFs), do not occur on a predictable basis and are not characterized by changes in behavior and physiology that follow annual patterns. Rather, these events cannot be anticipated and are not associated with anticipatory physiological changes (Wingfield et al. 1998). LPFs can be deleterious events such as El Niño (Steinfartz et al. 2007), acute predation (Monclús et al. 2008), and severe storms (Clutton-Brock 1991). However, the definition of an LPF could be extended to include anthropogenic stressors, or unnatural stressors created by the undertakings of humans. The term "anthropogenic stressor" can encompass a wide variety of activities, including

exposure to pollutants (Barros et al. 2014), habitat modification (Deb et al. 2014), poaching (Gobush et al. 2008), or encounters with humans during critical life stages (Villanueva et al. 2012). An anthropogenic stressor could easily be thought of as an LPF because animals cannot predict the changes caused by anthropogenic stressors, and they have not evolved to mediate their effects via seasonal changes in physiology. Thus, anthropogenic stressors can have especially detrimental effects on wildlife.

Many biologists are becoming increasingly aware that human-induced anthropogenic stressors, such as encounters with humans during critical life stages, can have deleterious effects on wild animals. The consequences of anthropogenic stressors are often investigated through a black and white filter; they either cause a given wild species to go extinct or they do not. For example, researchers have examined the effects of urbanization on species richness within certain groups, such as subtropical evergreen broad-leafed forest vegetation (Huang et al. 2013) and rove beetles (Magura et al. 2013). In many of these studies, the species that survive do so because they have certain traits that render them suitable to life in an urban environment. For example, tall, subtropical evergreen forest plants possessing traits including tolerance to soil acidification were better able to persist in urban forests than low-lying, clonally dispersing plants (Huang et al. 2013). These kinds of studies categorize which species can persist despite anthropogenic stressors and which cannot. However, they do not address the quality of life of those species that do manage to persist. One may then ask the question, *does survival equal prospering?* It is possible that even if a species persists despite the presence of anthropogenic stressors, individuals of the species experience sub-lethal effects that may influence their fitness. Indeed, these sub-lethal effects have the potential to be more detrimental to population-level fitness because, unlike lethal stressors, which immediately remove the deleterious phenotype

from the population, sub-lethal fitness effects have the potential to reduce the reproductive output of living individuals in the population (Sheehan 1984, Edge et al. 2013, Wilson et al. 2014). Additional studies are needed that address these critical questions for a variety of taxa.

In the biological community, there is a growing recognition of the importance of measuring sub-lethal effects of anthropogenic stressors. Indeed, an expanding literature addresses these topics in birds (Taylor et al. 2014, Giraudeau et al. 2014, Strasser and Heath 2013), mammals (Vick et al. 2012, Baria et al. 2007, Pereira et al. 2006), and reptiles (French et al. 2010, French et al. 2008). These studies have shown that, although certain species of animals have adapted or acclimated to human-modified landscapes, these species often exhibit altered stress hormone levels. For example, copperhead snakes (*Agkistrodon contortrix*) living in habitat cut by busy roads exhibit a dampened stress response in comparison to copperhead snakes living in habitat with few roads that are not heavily traveled (Owen et al. 2014). Results such as this could indicate that prolonged chronic stress associated with anthropogenic factors, such as busy roads, alter the stress physiology of wild animals. In this example, busy roads appear to have inhibited the ability of copperhead snakes to mount an adequate stress response. Thus, wild populations living near busy roads, cities, or other modified habitats, may be more likely to be depredated or succumb to other perils.

Conservation science arose as a field in response to the emerging crisis of global biodiversity loss (Soulé 1986). The complexities associated with biodiversity loss and population declines has necessitated that conservation science become a multi-disciplinary field with diverse areas of specialization, including conservation genetics (Frankham 1995), conservation behavior (Sutherland 1998), conservation social science (Mascia et al. 2003), and others. Each of these sub-disciplines of conservation science strive to measure certain characteristics of populations

and how they will respond to human-induced changes (Lennox and Cooke 2014). Physiological knowledge has been used for many years in conservation science. Perhaps the most famous success story in the sub-discipline of conservation physiology in the United States was identifying the effects of dichloro-diphenyl-trichloroethane (DDT) on reproductive biology in raptors and other apex predators. This discovery led to the ban of DDT and DDT-like substances in large parts of the world (Wikelski and Cooke 2006). Physiological knowledge adds a useful tool to the conservation biologist's toolkit because it can help identify optimal ranges in habitat (and therefore predict range-shift in response to climate change) (Tomanek 2010; Somero 2012; Telemeco and Addis 2014), how human activities affect hormone levels in wild populations (Lennox and Cooke 2014; Wikelski and Cooke 2006), and so on. As different hormones have distinct, important effects on behaviors such as reproduction, understanding how animal physiology is affected by anthropogenic stressors can help answer critical conservation questions such as how populations will change as anthropogenic stressors continue, or whether populations are likely to persist at all in the face of human encroachment (Cook et al. 2013, Wikelski and Cook 2006).

For my dissertation, I measured CORT to clarify organismal responses to stress. CORT is a well-recognized stress hormone that has been studied successfully in wild reptiles (Palacios et al. 2012, Robert et al. 2009, Rodl et al. 2007). Measures of CORT provide insights into the activation of the hypothalamus-pituitary-adrenal (HPA) axis. This major neuroendocrine system maintains energy balance and modulates energy allocations during and after encounters with stressors (Sapolsky et al. 2000). Specifically, CORT interacts with two membrane-bound receptors that move to the nucleus and act as transcription factors (Romero and Wingfield 2016). The mineralocorticoid receptor (MR) is present at high densities within the cell, while the

glucocorticoid receptor (GR) is present at lower densities (Sapolsky et al. 2000). Additionally, a lower-affinity MR in the plasma membrane binds to CORT when it is circulating at high concentrations. This MR mediates nongenomic cellular responses in neurons of the hypothalamus and hippocampus and can produce rapid behavioral responses (de Kloet et al. 2008, Romero and Wingfield 2016). At basal levels, CORT primarily binds with nuclear MRs (for which it has a higher affinity), but at increased stress-induced concentrations CORT also binds with GRs and plasma membrane MRs to mediate various aspects of the stress response (de Kloet et al. 2008). These include keeping the animal in a heightened state of awareness after experiencing an acute stressor, promoting recovery from stress, and re-establishing homeostasis (Bradshaw 2003, Romero 2004). CORT in blood plasma is bound to corticosterone-binding globulins that regulate the amount of CORT available to tissues (Breuner et al. 2013). Thus, the proportion of bound versus unbound CORT is important in determining the downstream effects of the stress response (Edwards and Boonstra 2016). Considering these factors, measures of total CORT must be interpreted carefully. Nevertheless, many studies have shown significant associations between circulating levels of total CORT and stress-related physiological and behavioral phenotypes (Moore and Jessop 2003, Robert et al. 2009, Vitousek et al. 2014). Therefore, measuring total circulating CORT can provide insight into physiological state and responses to stressors, especially when combined with additional indicators of physiological response (Schoech et al. 2013).

Physiology provides the mechanism by which organisms perceive environmental cues and respond to them readily. Thus, physiology contributes to the phenotype of wild animals by regulating both how organisms respond to annual, predictable events such as reproduction and migration, as well as how they respond to perceived threats (Romero and Wingfield 2016).

However, physiological responses can be difficult to interpret, and it is important to measure fitness-related traits at behavioral and morphological levels to thoroughly understand how wild animals react to perceived threats or annual predicted events. Thus, it is key to assess fitness-relevant morphological traits and behaviors, as well as physiological measures, when attempting to understand how organisms respond to perceived threats.

When trying to evaluate how organisms will respond to rapidly changing environmental conditions, perhaps due to habitat loss or climate change, it is also important to measure transgenerational effects. In addition to determining when and where offspring will be produced, a mother's experience of the environment may degrade her body condition or alter her physiological state (for example, exhibiting heightened circulating stress hormones). Mothers may in turn transmit these experiences to their offspring via cytoplasmic elements such as lipid, protein, hormone, or mRNAs present in the yolk. These factors may then influence offspring development and eventual offspring phenotype (Mousseau and Fox 1998). In many cases, these transgenerational effects are adaptive. They may work to mitigate the potential mismatch between the anticipated and the realized environment by basing offspring phenotype, including physiology, behavior, and morphology, on the conditions experienced by recent ancestors (Raubenheimer et al. 2012). Thus, transgenerational effects can be viewed as an extension of plasticity across generations (Bonduriansky et al. 2012). However, one transgenerational effect, elevated levels of maternal CORT, can have negative consequences for offspring fitness, including reduced immune function in yellow-legged gulls (Rubolini et al. 2005), reduced reproductive success of female offspring in zebra finches (Naguib et al. 2006), and decreased offspring length at hatching in reef fishes (McCormick 1999, 2006). Thus, it is important to

consider if the form of non-genetic inheritance enables the population's mean phenotype to track a rapidly changing environment (Bonduriansky et al. 2012) or if it is largely destructive.

There is also the possibility that transgenerational effects are nuanced and context-dependent. That is, in certain situations or to varying degrees, they are beneficial, but in other situations or to extreme degrees, they are detrimental to offspring fitness. For example, organisms exposed to stressful environments can produce offspring with traits that are functionally adapted to the conditions experienced by the parent. In *Polygonum persicaria*, parental plants raised in nutrient poor conditions produced offspring with significantly longer root systems than offspring of *P. persicaria* grown in nutrient rich soil (Sultan 1996). These longer root systems were beneficial in that they maximized root uptake surface for mineral ions (Wulff 1986). However, maternal effects are not necessarily adaptive, or they may only be adaptive in certain situations. For example, in the marine bryozan *Bugula neritina*, a common environmental stressor is the pollutant, copper. Mothers exposed to this pollutant produced offspring that were larger, better able to disperse, and more resistant to stress induced by living in copper-contaminated water. However, these effects were most noticeable in the larval stage of development; post-metamorphic *B. neritina* from copper-stressed moms exhibited rapid declines in performance compared to controls and fared poorly in intraspecific competition (Marshall 2008). Finally, it is not always clear how offspring phenotypes will be affected in species that have not been previously measured. For example, in *P. persicaria*, drought-stressed parents produced offspring with longer, more quickly growing root systems. In contrast, offspring from drought stressed parents of the closely related *P. hydropiper* (typically restricted to moister habitats) exhibited reduced, slowly growing root systems (Sultan et al. 2009). Thus, additional

research striving to understanding how species differ in this element of transgenerational plasticity will help elucidate organismal response to changing climate or anthropogenic stressors.

For my Ph.D., I wanted to extend the existing literature by examining the effect of stressors (including anthropogenic stressors) on stress hormone levels in a relatively unstudied taxon, freshwater turtles, exemplified by the painted turtle (*Chrysemys picta*). The painted turtle is common in still bodies of water across North America. Indeed, it is the only turtle whose range extends from coast to coast, and it is also found as far north as Ontario and as far south as northern Mexico. Over its expansive range, *C. picta* is exposed to a wide range of environmental conditions, food sources, and predation pressures, including close-proximity to human populations, as well as in largely undisturbed areas (Ernst and Lovich 2009). This abundance of populations facilitates comparisons across a spectrum of more human-impacted to less human-impacted localities. One well-studied population of *C. picta* is found at Thomson Causeway Recreation Area (TCRA) on an island in the Mississippi River. This population of painted turtle has been studied continuously since 1988 (Schwanz et al. 2010, Refsnider and Janzen 2016), providing a powerful backdrop of natural history knowledge and experimental findings to instruct my research into the effects of anthropogenic stressors and transgenerational stress.

Previous research on freshwater turtles has shown that these vertebrates can exhibit detrimental effects of anthropogenic effects, such as elevated levels of mercury in blood and tissue (Chaffin et al. 2008), or decreases in genetic diversity in populations that have been overharvested for food (Mali et al. 2015; Gonzalez-Porter et al. 2011). However, interactions with humans are not always negative. Painted turtle nesting behavior is not affected by human recreational activity (Bowen and Janzen 2008). Furthermore, turtles that do nest next to anthropogenic structures actually may experience a decrease in nest predation (Strickland and

Janzen 2010). Fewer studies have documented the effects of human disturbances on behavior and physiology in wild freshwater turtles. However, animals in disturbed sites bask for shorter durations and exhibit high heterophil to lymphocyte levels, indicating these turtles experienced increased stress (Selman et al. 2013). Additionally, yellow-blotched map turtles (*Graptemys flavimaculata*) commonly abandon their nests and basking turtles flee into the water upon approach of a boat (Moore and Seigel 2006). Furthermore, animals that flee can take up to an hour to return (Moore and Seigel 2006). Considering the important role that nesting and basking play in turtle metabolism, immunocompetence, vitellogenesis, and population level recruitment, this study indicates a very real threat to turtle populations if they all react as strongly to human disturbances.

The studies presented here provide an integrative approach toward elucidating how wild reptiles react to anthropogenic stressors and the transgenerational effects of elevated stress (anthropogenic or otherwise). By measuring physiology, as well as morphology and behavior, we enhance our understanding of how these traits covary in a wild reptile and further our understanding of how wild reptiles react to stressors. These experiments fill an important knowledge gap connecting physiological, behavioral, and transgenerational responses to elevated stress in an understudied taxon, freshwater reptiles. In addition, climate change scenarios forecast increased frequencies of extreme events (Solomon et al. 2007), and turtles around the world already suffer substantially from reduced availability of viable habitat (Böhm et al. 2013). These pressures may increase stress levels, even in species without high aptitude for adapting to human-modified landscapes. Thus, sub-lethal effects of anthropogenic stressors and transgenerational effects of maternal stress may play important roles in population viability of turtle species. This research helps clarify how freshwater turtles are reacting to their rapidly

changing world. I hope these contributions will serve as a strong addition to the rich history of ecological, behavioral, physiological, and conservation-oriented studies in freshwater turtles.

Dissertation Organization

Chapter 1: Stress hormone levels in a freshwater turtle from sites differing in human activity

Glucocorticoids such as CORT commonly serve as a measure of stress levels in vertebrate populations. These hormones have been implicated in regulation of feeding behavior, locomotor activity, body mass, lipid metabolism, and other critical behaviors and physiological processes. Thus, understanding how glucocorticoids fluctuate seasonally and in response to specific stressors can yield insight into organismal and overall health of populations. I compared circulating CORT levels between two similar populations of painted turtle, *Chrysemys picta*, which differed primarily in level of exposure to human recreational activities. I measured basal CORT levels as well as the CORT stress response, and did not find any substantive difference between the two populations. This similarity may indicate that painted turtles are not stressed by the presence of humans during the nesting season. The results of this study contribute to our understanding of CORT levels in freshwater reptiles, a historically underrepresented group. They also give insight into how these species may respond to human recreational activities during critical life stages, such as the nesting season (Polich 2016)

Chapter 2: Flight initiation distance in a freshwater turtle, *Chrysemys picta*

Many wild animals are subjected to stressors in the form of direct human disturbances from recreational activities. However, for many species it is unknown how these stressors affect

individual fitness and behavior. We tested whether *C. picta* would allow closer human approach, as measured by flight initiation distance (FID), at a site with regular presence of humans as compared to turtles less exposed to humans. Painted turtles regularly exposed to the presence of humans had significantly shorter average FIDs compared to those at a non-recreation site. The ability of turtles to tolerate closer human approaches in recreational areas may allow turtle populations to coexist in the presence of some human activity (Polich and Barazowski 2016).

Chapter 3: Effects of augmented stress hormones on painted turtle development and behavior

Maternal stressors can play an integral role in offspring development and ultimate behaviors in various vertebrates. Elevated circulating stress hormones can be reflected in elevated concentrations in ova, thus providing a potential mechanism for maternal stress to be transmitted to offspring even in taxa without parental care. In this study, we assessed the potential impacts of augmented stress hormones on offspring development and anti-predator behaviors in *C. picta*. We exposed *C. picta* eggs to biologically relevant amounts of CORT, as a proxy for maternal stressors. We allowed the eggs to incubate in the field, then measured offspring phenotypes, conducted performance trials, and simulated nest emergence in a field experiment. Exogenous CORT reduced survivorship to hatch, but did not affect incubation duration, offspring size, overwinter survival, or size after hibernation. In performance trials, this hormone treatment reduced the frequency of righting, yet enhanced the righting speed of neonates. Regardless, these performance differences did not translate to altered survivorship in the nest emergence experiment. These results are relevant to understanding the potential effects of maternal stress levels on offspring phenotypes, as well as the robustness of offspring fitness to altered levels of maternal stress in freshwater turtles.

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CHAPTER 2

FLIGHT INITIATION DISTANCE IN A FRESHWATER TURTLE, *CHRYSEMYS PICTA*

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Abstract – Many wild animals are subjected to anthropogenic stressors in the form of direct human disturbances from recreational activities. However, for many species it is unknown how these stressors affect individual fitness and behavior. We tested whether painted turtles, an imperiled freshwater species, would allow closer human approach, as measured by the flight initiation distance (FID), in the regular presence of humans as compared to turtles not exposed to humans. We collected data at a site closed to recreation and one with regular recreation and found that painted turtles regularly exposed to the presence of humans had significantly shorter average FIDs compared to those at the non-recreation site. The ability of turtles to tolerate closer human approaches in recreational areas may allow turtle populations to coexist in the presence of some recreational uses.

Introduction

Turtles are unique and recognizable vertebrates. With over 300 extant species and residence on every continent except Antarctica (van Dijk et al. 2014), they have been incorporated into many human cultures as a source of food, tools, and inspiration for folklore and creation myths. Turtles are also an ancient taxon, with representatives that evolved shelled forms appearing in the fossil record over 200 million years ago (Ernst and Lovich 2009). Despite the apparent success of turtles as an enduring taxon and the high regard in which most cultures hold these animals, turtles today are in serious decline, with over half the species threatened with extinction (van Dijk et al. 2014). Many factors precipitating these declines are similar to those faced by all animals and include habitat loss and degradation, unsustainable exploitation for food markets and the pet trade, environmental pollution, and global climate change. However, the argument can be made that these stressors are amplified in turtles because they generally rely on individual longevity to maintain healthy populations (e.g., Congdon et al. 1994).

We examined how adult painted turtles (*Chrysemys picta*) responded to the presence of humans during a critical life stage, the nesting season. Can wild *C. picta* accommodate the presence of humans or do turtles perceive humans as a continual predation threat? Many wild vertebrates respond to human-disturbance stimuli as they would to predation attempts (Frid and Dill 2002), but some species can habituate to human presence (Ellenberg et al. 2009, Selman et al. 2013). In the wild, basking is an important and widely-displayed behavior in freshwater turtles that could be affected by human disturbances. Turtles that cannot accommodate human presence may decline in health (Moore and Seigel 2006). With human populations growing, more turtle populations experience contact with humans throughout their life cycles. Therefore, knowing whether or not freshwater turtle species can tolerate human presence can provide useful

insight into whether these species will be able to persist in their native habitats despite human encroachment.

Chelonians have numerous predators. This is especially true of hatchling and juvenile turtles (e.g., Janzen et al. 2000), but may occur at any life stage (Greene 1988). In response, chelonians have evolved a multitude of physical and behavioral defenses, including retreat into their iconic shells. Other anti-predator behaviors emphasize crypsis, cloacal discharge, postural threats, and biting (Greene 1988). Of particular note is the fact that basking freshwater turtles often flee into nearby water to avoid predation. Similar escape behaviors have been studied in other taxa, particularly birds (Tarlow and Blumstein 2007; Blumstein and Fernández-Juricic 2010). There is the potential for wild animals to perceive human recreational activities as predation attempts. They may not have evolved the means to appropriately measure the threat levels of various human recreational activities. Thus, they may overreact to perceived anthropogenic threats, diverting resources to immediate survival and away from important life-history components such as reproduction. It is therefore critical to measure and understand how animals react to potential anthropogenic stressors, including human recreational activities.

The response of animals to human approach is often measured by flight initiation distance (FID), which is the distance to which a putative predator can approach a wild animal before the animal flees (Blumstein 2003; Cooper 2008, 2009; Schwanz et al. 2011). FID is highly germane to freshwater turtles because of its relationship to the basking habit, which is linked to regulation of metabolism, decrease in parasite load, and egg deposition (Ernst and Lovich 2009). Although basking is beneficial for freshwater turtles, it is potentially dangerous as it leaves the animals more vulnerable to predation. Indeed, many freshwater turtle species rarely emerge from the water except to nest or bask on supra-aquatic surfaces (Ernst and Lovich 2009;

Steen and Gibbs 2004). Thus, an estimate of how sensitive basking animals are to disturbance by humans can give insight into adverse impacts on important life-history processes.

We compared the average FID in two groups of painted turtles, one exposed to recreation and one not experiencing recreation, for which raccoons (*Procyon lotor*) and otters (*Lontra canadensis*) were the only known non-human predators on adults (F. Janzen, pers. obs.). We hypothesized that painted turtles at the site with frequent recreation activity would perceive humans as less of a threat and have a shorter average FID, compared to painted turtles at a field site with minimal human activity (*sensu* Bowen and Janzen 2008; Spencer and Janzen 2010; Strickland et al. 2010 for nesting behavior).

Methods

The two field sites were approximately 32 km apart along the Mississippi River in northwest Illinois (Figure 1). The first population was located at Lost Mound Unit (hereafter rural), an ex-military base that has long been off-limits to human recreational activities. Although this site was used to fire artillery, by 1920 it served primarily as a munitions storage and recycling facility. As the munitions storage facilities were located upland and the base had few personnel present for many years, the turtle population in the river had not been exposed to many humans for over a century. The other population is found at Thomson Causeway (hereafter urban), a popular camping and fishing area that experiences substantial human activity. For example, 7301 campers visited the area from 31 March to 31 October 2013, a figure that does not include additional day visitors (K. Zidarich, pers. comm.). The type of human activity to which a given turtle was likely exposed was based on whether the turtle was engaged in aquatic or terrestrial activity. Most of the turtles that emerged onto the land during the time period in which this study was conducted were gravid females searching for a suitable nest site location or

actively nesting. These animals were likely to be exposed to human approaches on foot or approaches from vehicles. Alternatively, turtles engaged in aquatic activities such as aquatic basking were likely to be approached by on-shore fishermen or boats. Despite these various human activities, turtles at this site frequently nest in close proximity to humans (Bowen and Janzen 2008; Strickland et al. 2010).

For each field site, multiple locations to assess FID of adult painted turtles basking on logs were chosen based on ease of access and how well they represented basking locations within each site. At the urban site, 12 locations were observed that were close to popular campsites or near areas where people would regularly fish. At the rural site we observed 2 locations. Not only was human recreational activity precluded at this site, but most locations were strictly off-limits because of the potential for unexploded ordinance in the water. The locations at both sites were characterized by the presence of large logs in the water and therefore were frequented by basking painted turtles.

Every day from 26 May to 20 June 2013, one person approached the turtle(s), while a second person recorded FID. To control for climatic variables, such as daily fluctuations in temperature, FID trials were performed at both sites at the same time each day by two groups of two researchers. For consistency's sake, RLP was always the approaching partner at the rural site and MB was always the approaching partner at the urban site, targeting turtles from the same chosen spot at each location every time FID was measured. Starting distance was standardized for each location within each site such that a researcher always approached basking turtles at a given site from the same standardized distance, but the distances varied based on location and site. At our rural site, starting distances were 47 and 109 m for two sites. At our urban site, starting distances ranged between 36 and 125 m for 12 sites. The recorder used a laser-range

finder (model: Nikon Prostaff 550, 0.0144-m accuracy from 91–503 m) to obtain the initial distance between the researcher and a turtle, and the distance between the researcher and the turtle when it fled. For the second measurement, in instances where multiple turtles fled from the log at once, we conservatively recorded only a single FID measurement taken from the center of the group of basking turtles. In instances when some turtles fled but others did not, the researcher continued to approach the turtles and, each time a new group of turtles fled, a new FID was recorded. Evidence from a basking study that occurred simultaneously with this one and that involved trapping and marking individuals suggests that individual turtles infrequently returned to the same spots to bask day after day (RLP, unpubl.). Thus, we expect that we rarely sampled the same turtles more than once.

We analyzed the FID data with SAS software (SAS 9.4, 2015). We performed a nested analysis of covariance (ANCOVA), using the model:

FID = date, time, site, site(location), date*site, time*site

where the covariates tested included "date," the day of the year on which an FID trial was performed; "time," the exact time (military time) of day that an FID trial occurred; "site," whether rural or urban; and "site(location)," the FID location within each field site. In this model, we also tested the interactions "date*site" and "time*site" to see if date on which FID was assessed or time of day at which FID was recorded had context-dependent impacts on FID.

Results

Over 26 days, we collected 97 observations from two locations at the rural site and 238 observations from 12 locations at the urban site. The covariate "time" was statistically significant, as were the interactions "date*site" and "time*site" (Table 1). For the time*site interaction, turtles at the rural site exhibited decreased FIDs later in a given day. Overall, we

detected a significant difference in the approach distance tolerated by painted turtles in the two populations. Turtles at the rural site fled at greater distances than turtles at the urban site (Table 1). On average, painted turtles at the urban site fled at 40.7 m ± 1.451 (SE), while painted turtles at the rural site fled at 60.0 m ± 8.218 (SE). The factors "date" and "site (location)" were not significant main effects (Table 1).

Discussion

We hypothesized that animals in populations where human presence is common would flee at shorter distances, showing greater tolerance of human activity, than those not regularly experiencing human approach. Our results are consistent with this hypothesis. We found that adult painted turtles basking on logs where human disturbance was not common did flee at much greater distances than those at the more regularly human-visited site. By presumably adjusting FID based on how often they were exposed to humans and, therefore, on the threat-level that they perceived from humans, the more urban population of painted turtles minimized the loss of critical basking opportunities that fleeing more frequently would have incurred.

This FID information is especially relevant because turtles disturbed from their aquatic basking sites can take up to an hour to return (Moore and Seigel 2006). Therefore, if turtles are consistently frightened off logs, they could potentially lose significant amounts of basking time. A reduction in basking could decrease fitness due to inability to thermoregulate and perform critical reproductive and self-maintenance tasks, including regulation of vitellogenesis (which may be especially crucial during the nesting season, when we conducted this study), as well as proper regulation of metabolism and control of parasite loads (Ernst and Lovich 2009). Indeed, turtles in poor health condition, as measured by higher parasite loads and lower white blood cell

counts, may spend more time basking than their peers in better health, suggesting that turtles may actively bask as a mechanism to improve their immune system (Ibáñez et al. 2014).

Why did *C. picta* at the urban site exhibit a shorter FID than painted turtles at the rural site? This study did not examine all possible explanations for this difference, but one potential explanation is that the turtles at the urban site learned via habituation (Rodriguez-Prieto et al. 2007) that most humans are not a threat to them while the turtles are basking. Habituation is often considered a learning process; as animals are repeatedly exposed to a putative stressor, they eventually cease to regard it as dangerous. Such flexible behavioral capacity may help turtle populations survive in the presence of humans whose behavior is not directly harmful or lethal to turtles. Another possible explanation relates to turtle size. Ibáñez et al. (2014) found that the amount of time male Spanish terrapins (*Mauremys leprosa*) hid in their shells after predator attacks depended on size, with larger animals spending more time hidden before emerging. If the turtles at our two study sites differed in size, this could be a factor in the difference in FID between the two *C. picta* populations. Turtle health is another possibility. Polo-Cavia et al. (2009) studied western pond turtles (*Emys marmorata*) residing in two populations in northern California. One population resided in a pristine, un-altered habitat. The other was found at a site with altered habitat where turtles were potentially exposed both rural and urban anthropogenic water contaminants. This study found that turtles residing in the altered habitat (higher concentrations of aquatic contaminants) had lower immunocompetence compared to their conspecifics residing in an unaltered habitat (Polo-Cavia et al. 2009). Since turtles at both sites for this study shared the same watercourse, we assumed that both populations were exposed to the same contaminants and therefore experienced the same impacts.

It is also possible that the *C. picta* at our urban site exhibited a shorter FID than the *C. picta* at our rural site due to differences in starting distance for our FID trials at the different locations within each site. This effect has been studied within the flight initiation distance literature, particularly in birds (Blumstein 2003) and lizards (Cooper 2005, 2008). However, we think it is unlikely that the results we observed in this study were due to an artifact of starting distance. This is due to the fact that the starting distances for the two locations within our rural site fell within the range of those that we used for our 12 urban sites. Thus, if starting distance had a statistically significant effect, we should have seen that effect in our ANCOVA when we tested for an effect of location.

Our results showed that, although rural turtles had a larger FIDs than the urban animals overall, flight distances were especially large in the morning when turtles were cold. Turtles allowed us to approach more closely before fleeing during the warmer part of the day, perhaps because, when temperatures rise, turtles may gain confidence in their ability to escape and thus allow potential threats to approach closer before fleeing. This effect has been noted in other ectothermic vertebrates including *Anolis* lizards, tropical whiptail lizards, and brown trout (Cooper 2006, Lattanzio 2014, Öhlund et al. 2014). It is important to understand how other important factors influence the way turtles respond to predation threats, such as approaching humans. We also found a date*site effect, but this finding is likely attributable to the scarcity of painted turtles at the rural site at the beginning of this study.

If painted turtles, in general, can tolerate close human approach when regularly exposed to non-threatening human approach, this response may allow painted turtle populations to persist in the presence of certain recreational uses. Managers should collect more data on the response of basking turtles to different types of human approaches while including important

environmental factors in order to understand the types of recreation that pose the lowest threat to species that depend on basking.

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Table 1. ANCOVA table for full flight initiation distance model with sums of squares (SS), degrees of freedom (df), mean squares (MS), *F*-values, and *p*-values. Significant terms are printed in bold.

	SS	**df**	**MS**	***F***	***p***
Site	**6227.683**	**1, 1**	**6227.683**	**6.09**	**0.014**
Site(location)	14998.899	12, 12	1249.908	1.22	0.268
Time	**9631.461**	**1, 231**	**9631.462**	**9.43**	**0.002**
Date*Site	**16498.269**	**1, 24**	**16498.27**	**16.15**	**<0.0001**
Time*Site	**8495.176**	**1, 230**	**8495.176**	**8.31**	**0.004***
Date	691.247	1, 25	691.247	0.68	0.412
Error	225823.817	221	1021.827		

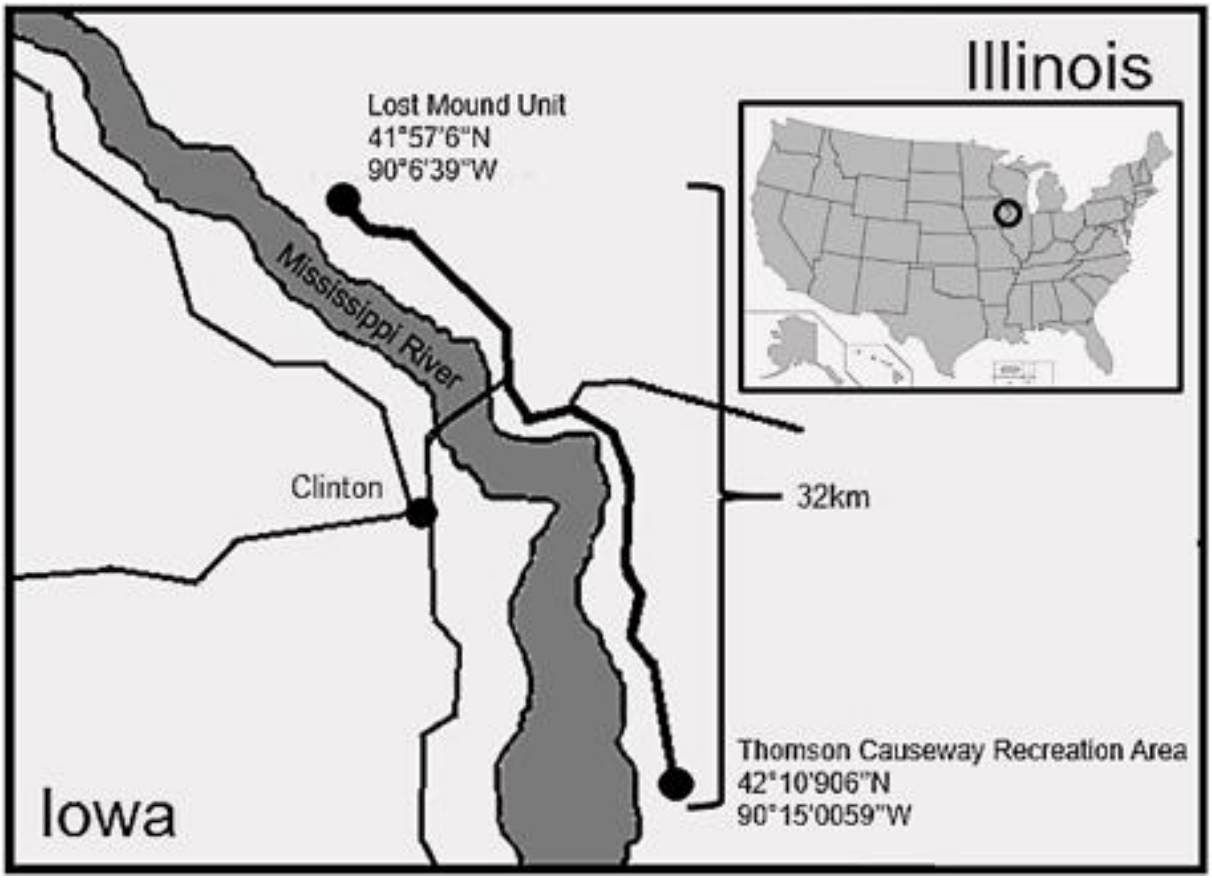

Figure 1. Location of the two study sites, Thomson Causeway Recreation Area (urban site) and Lost Mound Unit (rural site), along the Mississippi River in Illinois.

CHAPTER 3

STRESS HORMONE LEVELS IN A FRESHWATER TURTLE FROM SITES DIFFERING IN HUMAN ACTIVITY

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Rebecca L. Polich

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Abstract – Glucocorticoids such as corticosterone commonly serve as a measure of stress levels in vertebrate populations. These hormones have been implicated in regulation of feeding behavior, locomotor activity, body mass, lipid metabolism, and other critical behaviors and physiological processes. Thus, understanding how glucocorticoids fluctuate seasonally and in response to specific stressors can yield insight into organismal and overall health of populations. I compared circulating corticosterone (CORT) levels between two similar populations of painted turtle, *Chrysemys picta*, which differed primarily in level of exposure to human recreational activities. I measured basal CORT levels as well as the CORT stress response, and did not find

any substantive difference between the two populations. This similarity may indicate that painted turtles are not stressed by the presence of humans during the nesting season. The results of this study contribute to our understanding of CORT levels in freshwater reptiles, a group that is historically underrepresented in studies of circulating hormone levels. Specifically, studies that seek to use circulating levels of stress hormones such as CORT as a measure of the effect of human activities on wild populations. They also give insight into how these species as a whole may respond to human recreational activities during critical life-history stages, such as the nesting season. Although there was no discernable difference between circulating CORT levels between the urban and rural populations studied, I did find a significant difference in circulating CORT levels between male and female *C. picta.* This important finding helps us to better understand sex differences between male and female painted turtles, and adds to our understanding of this species and other species of freshwater turtle.

Introduction

Biologists have begun to recognize the importance of measuring the sub-lethal effects of anthropogenic stressors and a growing literature exists that addresses this topic in birds (Taylor et al. 2014, Giraudeau et al. 2014, Strasser and Heath 2013), mammals (Vick et al. 2012, Baria et al. 2007, Pereira et al. 2006), and some reptiles (French et al. 2010, French et al. 2008). Many of these studies have demonstrated that, although certain species have adapted to human-modified landscapes, they often display altered stress hormone levels. Stress hormones such as glucocorticoids are involved in regulating a number of vital behaviors, such as locomotor activity and feeding, as well as metabolic processes such as lipid metabolism. As such, they are believed to be crucial mediators of a suite of behaviors and physiological adaptations that can change with annual, expected events, such as breeding (O'Reilly and Wingfield 2001) and migration

(Ramenofsky and Wingfield 2007), or acute, unpredictable stressors, such as El Niño (Steinfartz et al. 2007), acute predation (Monclús et al. 2008), and severe storms (Clutton-Brock 1991).

Anthropogenic stressors can be considered chronic or acute depending on the regularity with which they occur. These human-induced stressors are particularly detrimental to wild vertebrates because it is unlikely that these organisms have evolved means to measure and mediate their effects via seasonal changes in physiology or via the glucocorticoid stress response. For example, human alterations to the environment could create situations where the glucocorticoid stress response is mismatched with the level of threat present. In this way, vertebrates may underreact to the threat, which could lead to death, or overreact to it, diverting resources to immediate survival and away from other life-history components such as reproduction (Angelier and Wingfield 2013). Such chronic overreactions to stressors can induce long-term fitness costs such as decreased immune system functioning, suppression of growth, severe protein loss, and inhibition of reproductive behavior (Busch and Hayward 2009). Thus, anthropogenic stressors can have especially detrimental effects on wildlife. For example, copperhead snakes (*Agkistrodon contortrix*) living in habitat cut by busy roads exhibit a reduced stress response in comparison to copperhead snakes living in habitat with lightly traveled roads (Owen et al. 2014). This result could indicate that prolonged chronic stress associated with living by busy roads has inhibited their ability to mount an adequate stress response. If so, then individuals in such circumstances may be more likely to be depredated or succumb to other perils, thereby decreasing their fitness.

Various studies also find direct evidence of human disturbances affecting important factors such as behavior, immune function, and fitness. For example, marine iguanas exhibit stress-induced decreases in immune function in populations exposed to anthropogenic

disturbances (French et al. 2010). This observed loss in immune function could induce population decline, as it is associated with increased parasite load and decreased ability to heal wounds. Similarly, elephants residing in areas of high poaching risk exhibited high fecal glucocorticoid levels and significantly lower reproductive output than elephants residing in areas of low poaching risk (Gobush et al. 2008). Thus, human stressors can affect wild populations at a number of different levels of biological organization. It is therefore critical to appreciate how human disturbances influence wild populations so that we can better understand future trajectories of these populations.

For this study, I examined the effect of human disturbance on stress hormone levels in a relatively unstudied taxon, turtles. Although endocrinological studies have been performed that examine circulating stress hormone levels (Cash et al. 1997, Hammon et al. 2002, Selman et al. 2012), this taxon is still underrepresented compared to birds, mammals, and other reptiles. I quantified circulating hormone levels in the painted turtle, *Chrysemys picta*, a small freshwater turtle that primarily occupies still bodies of water and can inhabit ponds in close proximity to human populations (Ernst and Lovich 2009). I compared corticosterone (CORT) levels in two wild populations of *C. picta* that differ primarily in the amount of human recreational activity occurring in the summer when the turtles are most active. One population was exposed to minimal human disturbance while the other was exposed to considerable human activity. I hypothesized that *C. picta* residing in environments that experience high amounts of human disturbance would exhibit higher baseline CORT levels or CORT stress responses than *C. picta* residing in environments that experience lower amounts of human disturbance. Testing this hypothesis will add to our understanding of how freshwater turtles react to human recreational activities by yielding insight into how these activities affect circulating stress hormone levels.

The nesting season, which occurs during part of the summer, is of particular importance to many freshwater turtle species because it a time of high fitness import for both male and female turtles. The nesting season is when eggs are laid, thus offspring production, a major component of organismal fitness, takes place during the nesting season. This is also when turtles are most likely to come into direct contact with humans (turtles bask frequently during the nesting season and females must emerge onto the land to lay their eggs).

Methods

Study populations

The study populations of *C. picta* are located ~32 km apart in northwestern Illinois on the Mississippi River. The two populations differ primarily in the amount of human recreational activity to which they are exposed. The urban site, Thomson Causeway Recreational Area (TCRA; 41°57’ N, 90°07’ W)(Schwanz et al. 2010), is a popular RV campsite and daytime recreational use area. For example, in 2013 when this study was conducted, over 7300 campers utilized this site from 31 March to 31 October (this number does not include the number of people who utilized this site as a daytime recreational area during that summer)(personal communication from Kevin Zidarich, U.S. Army Corps of Engineers). Still, *C. picta* frequently nest in close proximity to humans at this site (Bowen and Janzen 2008, Strickland et al. 2010). The rural site, Lost Mound Unit (LMU; 42º27’N, 90º39’W), is an ex-military base. Military usage of this site peaked in 1945, with the number of personnel declining to 400 by 1995, five years before the site officially closed in 2000. As the munitions storage facilities are located upland and the base had few personnel present for many years, the turtle population in the river has not been exposed to sizable amounts of human traffic for a considerable time.

In addition to the differences between these two sites in terms of terrestrial occupation by humans, they also differ substantially in aquatic usage by humans. For example, the portion of the Mississippi River backwaters flowing through LMU are off-limits to human recreational activities due to the historical practice of firing munitions over the river while the site was a military base. On the other hand, fishermen and other recreational boat users frequent the waters of TCRA throughout the summer. As daytime recreational use is not recorded at TCRA, exact numbers are not available for how many boats navigated the waters surrounding TCRA during the summers of 2012 and 2013. Although the two sites differ in aquatic and terrestrial usage by humans, they are fairly similar topographically. TCRA is located on an island in the Mississippi River close to mainland Illinois. The animals collected for this study were collected from a *C. picta* population that nests on the side of the island facing mainland Illinois, thus, these animals inhabit the backwaters of the Mississippi River. Similarly, the site at LMU was located on mainland Illinois, but was sheltered from the primary flow of the river by a series of islands close to the coast. Therefore, these turtles also inhabited the backwaters of the Mississippi River rather than the river itself. This is typical of *C. picta* as they tend to prefer to live in ponds and backwaters rather than directly on major, fast-flowing rivers such as the Mississippi.

Despite the differences between these two sites in terms of human recreational activities, the turtles inhabiting these sites are very similar in terms of genetic makeup. These two populations are part of a large clade within *C. picta* that spread into the central Great Plains and Rocky Mountain region after the retreat of the Laurentide ice sheets 20,000 years ago. This clade contains virtually no variation in mitochondrial DNA, indicating close genetic similarity between these two populations (Starkey et al. 2003).

Fieldwork

Fieldwork was conducted over the course of two years; however, in the summer of 2012 samples were only collected from TCRA. That summer, sample collection began on May 22 and ended on June 24. In the summer of 2013, fieldwork was conducted at both sites between 11 June and 20 July. Circulating CORT plasma samples were obtained from turtles that had just finished nesting and also from free-ranging male and female turtles captured while basking on logs. Basking turtles were captured using aquatic basking traps. These basking traps were constructed from four pieces of lumber (2”x4”) nailed together. A strip of fine mesh was attached to each board and formed a hoop at the bottom of the basking trap. This trap was then tied to an aquatic log on which painted turtles had previously been observed basking. Basking turtles were approached at a walk until they fled into the water in the middle of the basking trap. The turtle was then collected from the basking trap and a blood sample was quickly taken. If it took over three minutes to complete the approach and take the blood sample, that blood sample was not used in this study. These traps float at the surface of the water and capture turtles that flee into the water to escape perceived threats. At the urban field site, six males and one female were obtained through usage of the basking traps. At the rural site, 21 males and 11 females were obtained using basking traps.

I collected blood samples (0.25-0.5cc) for baseline and stress-induced CORT analyses from the urban site in both 2012 and 2013, and from the rural site in 2013 (Table 1). I obtained basal blood samples within three minutes of capture from the caudal vein at the base of the tail using heparin-rinsed syringes and considered those samples to represent baseline levels (Refsnider et al. 2015). I separated plasma from red blood cells via centrifugation, snap-froze the plasma in liquid nitrogen, and stored it at -80°C until analysis. I collected samples for stress-

induced CORT analyses following a standard capture-restraint protocol similar to that utilized in many vertebrate studies to activate the adrenocortical axis (Aguirre et al. 1995, Cash et al. 1997, Cash et al. 2005, Jessop et al. 2004, Palacios et al. 2012, Wingfield and Ramenofsky 1999). Turtles were placed in plastic buckets and collected additional blood samples at 10, 30, 50, 90, and 180 min after time 0. I obtained a body temperature after each blood sample was collected. All painted turtles used in this study, male and female from both sites, were confined in the same type of bucket and kept away from human activity in a shaded area. Thus, any stress associated with being confined would have been shared among all animals and would not have confounded the study. After blood collection was complete, I measured the plastron length for all turtles with a calipers. To account for natural sources of variation, I sexed all turtles based on fore claw length as well as tail length and shape, and overall body size: adult males have longer fore claws and more massive tails, but smaller body sizes than adult females.

Corticosterone radioimmunoassay

I assayed total plasma CORT using a double antibody radioimmunoassay (RIA) kit (Catalog # 07-120103, MP Biomedical, Orangeburg, NY) that has been validated in this study system (Refsnider et al. 2015). To assay total plasma CORT in *C. picta*, I used a modified version (Refsnider et al. 2015) of the MP Biomedical protocol for the I^{125} CORT RIA. I diluted the *C. picta* samples 1:20 with steroid diluent, this dilution having been shown to best stay within the standard curve concentrations for *C. picta* (Refsnider et al. 2015). To validate the use of this radioimmunoassay for *C. picta*, I tested for parallelism between the kit standards and the serial dilutions of a pool derived from my plasma samples (henceforth "plasma pool," generated from 5µl of plasma from five randomly-chosen individuals from each year). The resulting curve was validated as parallel to the standard CORT curve after logit transformation (painted turtle: slope

= -0.4473, R^2 = 0.9504, CORT standards: -0.4621, R^2 = 0.9834), confirming the validity of evaluating circulating CORT in *C. picta*) using this radioimmunoassay (Robert et al. 2009). The pool and the low controls provided in the CORT RIA kit served as internal controls. For the CORT stress response analysis, I analyzed repeated samples for each time point in the stress response series from a given individual in the same assay to minimize within-individual variation. Samples from different individuals from each population were randomly assigned to different assays using a random number generator (Palacios et al. 2012). In total, two assays were conducted. The first analyzed the *C. picta* plasma samples collected in 2012, the second analyzed the *C. picta* plasma samples collected in 2013. The first assay contained 11 individuals and 66 samples (basal and stress response series). The second contained 43 individuals and 153 samples (basal and stress response series). I calculated intra- and inter-assay coefficients of variation (CVs) of percentage bound of the internal controls to assess assay precision. Average intra-assay variation was 7.19% and average inter-assay was 14.44%.

Statistical analyses

I conducted all statistical analyses using the statistical program software SAS (SAS 9.3, SAS Institute Inc., Cary, NC, USA). For my analyses, I combined data from 2012 and 2013 at the urban field site to form a final CORT data set because a preliminary analysis detected no statistical difference in CORT levels between these two years. Before analysis, I performed a Shapiro-Wilk test, which indicated the CORT data set was not normally distributed. Therefore, I $\log_{10}$ transformed CORT levels to normalize the data. In addition, I removed one data point because it suggested a level of CORT higher than the highest concentration in the standard curve, and therefore could not be reliable, a known method of identifying outliers in hormone analyses (see Sparkman et al. 2014). Before conducting analyses of variance (ANOVA), I employed

Akaike's Information Criterion adjusted for finite sample sizes (AICc) as a model selection procedure to determine which independent variables I should include in my model. I conducted this analysis using the Proc GLMSELECT procedure in SAS, which performs effect selection in the framework of general linear models.

Based on model selection analysis, I evaluated baseline CORT using the following general linear model:

$$Y = \mu + \text{population} + \text{PL} + \text{sex} + \varepsilon$$

Where Y is the dependent variable (also response variable), μ is the mean of the distribution of samples, and ε is an error term typically included in general linear models. It contains the variability of the dependent variable (Y) not explained by the independent variables. In addition, population is urban vs. rural, PL (plastron length) is a common proxy for size in turtles, and sex is male vs. female. Other independent variables included in the AICc model selection analysis included time of day (TOD), body temperature (BT), date (month, day, year), capture method (via aquatic basking log or post nesting), assay (which RIA the sample was included in), as well as several interactions (PL*sex, date*sex, TOD*sex, and pop*sex). None of these variables were determined to be significant by the AICc model selection analysis, so I excluded them from the final model. I conducted the ANOVA procedure using the mixed-model procedure (Proc Mixed) in SAS software.

Similarly, I performed an AICc model selection analysis on my CORT stress response data to determine which independent variables I should include in that model. Based on this analysis, I analyzed stress-induced CORT using the repeated-measures general linear model:

$Y = \mu + \text{population} + \text{PL} + \text{sex} + \text{time} + \text{date} + \varepsilon$

As above, *Y* is the dependent variable (also response variable), μ is the mean of the distribution of samples, and ε is an error term typically included in general linear models. Additionally, population is urban vs. rural, PL is a common proxy for size in turtles, sex is the effect of male vs. female, time is the effect of the six repeated measures of CORT from the capture-restraint protocol, and date is when the sample was taken. Additional independent variables included in the AICc model selection analysis included time of day (TOD), body temperature (BT), method of capture, assay, as well as the interactions PL*sex, sex*time, sex*date, sex*TOD, and sex*pop. None of these variables were determined to be significant in the AICc model selection analysis, so I excluded them from the final model. After obtaining the model above, I conducted the ANOVA procedure using the mixed-model procedure (Proc Mixed) in SAS software.

In addition to the repeated-measures analysis for stress-induced CORT, I also performed statistical analyses using the area under the curve or integrated CORT response, the maximum CORT level achieved by each individual during capture-restraint protocol, the time required by each individual to reach maximum CORT level, and the amount of time required by each individual to descend to basal level after reaching maximum CORT levels. However, none of these analyses yielded different insights from those already obtained. Thus, I exclude these results for simplicity.

Results

Neither the baseline CORT analysis nor the CORT stress response analysis indicated a major effect of the independent variable of interest, population (stress-response CORT: $r^2 = 0.46$,

F = 0.41, P = 0.53)(baseline CORT: r^2= 1, F = 0.00, P = 0.97). The variable, sex, was important in both cases. Male turtles exhibited overall higher CORT at all time points, including baseline, compared to female turtles. Male *C. picta* had almost double the mean basal levels of plasma CORT (±s.e.), 35.77±6.33 ng/mL, than did female *C. picta*, 19.13±4.92 ng/mL (F = 8.68, P = 0.0049). For the capture-restraint protocol, male *C. picta* exhibited a higher overall stress response than did females (F=12.58, P=0.0009). For both basal CORT and stress response CORT, there was a trend for larger individuals to exhibit lower basal and stress response levels. However, in the basal analysis, PL fell short of being statistically significant (basal CORT: F= 3.08, P= 0.0857), while PL was statistically significant in the stress-response analysis (stress-response CORT: F=4.03, P= 0.0463).

In the repeated-measures analysis, time at which blood sample was taken also affected the levels of CORT measured. During the capture-restraint protocol, time point six (180 min) yielded the highest CORT concentration for males (85.87±33.2 ng/mL) and time point two (10 min) produced the highest CORT concentration for females (38.87±13.77 ng/ml) (Figure 1). However, the independent variable, date, did not affect CORT levels (F= 0.51, P = 0.47). The R-squared value for both of these mixed models (baseline CORT and stress-response CORT) was calculated by adding up the total sum of squares for the model terms and then dividing that number by the total sum of squares. In the case of the baseline CORT model, the model terms used to calculate sum of squares were Julian date, PL, sex, population, ID, and year. Total sum of squares was calculated by adding all of those terms as well as the residual. The final R-squared value was 1. For the stress-response-CORT model, the model terms used to calculate sum of squares were Julian date, PL, sex, population, time (which blood draw in the series the

sample came from), ID, and year. Total sum of squares was calculated by adding all of those terms as well as the residual. The final R-squared value was 0.46.

Discussion

As human populations continue to expand, wild populations must adapt to the new challenges they impose, or perish. Human encroachment does not necessarily result in complete loss of habitat. In some instances, such as National Parks, campgrounds, and recreation areas, native habitat may remain largely unchanged. However, wild species inhabiting these areas must nevertheless adapt to the presence of often very large numbers of people throughout the year or during specific time periods. In cases where wild populations persist despite human-induced stressors, it is still often unclear how healthy these populations are compared to populations living in relatively undisturbed habitats. I measured CORT levels in two wild populations of *C. picta*, one located at a highly human-impacted site and one located at a less human-impacted site, to gain insight into how freshwater turtles are affected by human recreational activities. I found no evidence that *C. picta* residing in highly human-impacted habitats exhibit higher stress levels than their counterparts living in less human-impacted areas. Interestingly, in a concurrent behavioral study performed by myself and colleagues on these same populations, we did find evidence that *C. picta* are able to modify flight initiation distance behavior based on levels of human recreational activity. The results of this study suggest that *C. picta* are able to habituate to the physical presence of relatively large numbers of humans (Polich and Barazowski, *in prep*).

I found no evidence to support the hypothesis that *C. picta* exposed to humans during the nesting season would exhibit heightened CORT levels, either baseline or stress-induced. If circulating CORT levels are a proxy for stress, this finding suggests that *C. picta* are not stressed by the presence of humans during the nesting season. Heightened CORT at the urban site would

have indicated that, compared to the rural population, the urban population is more stressed. As the urban and rural sites were ~32km apart on a similar stretch of the Mississippi River and exhibit virtually no genetic variation between one another (Starkey et al. 2003), this similarity in CORT levels implies either that the urban population has habituated to the presence of humans, or that *C. picta* are not stressed by the presence of human regardless of whether they have been exposed to them previously. This finding is in contrast to previous studies of songbirds and snakes (Lattin et al. 2014, Deviche et al. 2014, Moore et al. 2001). The results from these previous studies may differ from my own because *C. picta* are long-lived whereas most of the previously studied taxa are not. Evolutionary theory and some previous research has demonstrated that physiological as well as behavioral plasticity may help long-lived organisms, such as *C. picta*, survive dramatic environmental shifts (Bruno & Edmunds 1997; Dickinson et al. 1991; Levins 1968; West-Eberhard 1989). This would be advantageous to long-lived organisms because they have long generation times and may not respond to environmental perturbations rapidly enough if they rely on evolution alone. It is also possible that *C. picta* is a remarkably plastic species even for a long-lived vertebrate, and this plasticity has contributed to its incredible success as a species. For example, *C. picta* are plastic in nest-site choice behavior, resulting in similar incubation regimes and nest sex ratios across the vastly different climates that this species inhabits (Refsnider and Janzen 2012). This ability to modify behavior and physiology in response to a variable climate likely has led to the success of *C. picta* as a wide-ranging species, and may help it adapt to climate-warming scenarios (McGaugh et al. 2010). It is also possible that *C. picta* simply are not stressed by the presence of humans. Thus, even a naïve population of *C. picta* that has never been exposed to human disturbances would not exhibit

different levels of circulating CORT compared to a population that has never been exposed to human disturbance.

It is possible that a stress difference does exist between these populations and my methods were unable to detect it. For example, I collected more female than male samples at the urban site (total female = 39, total male = 10) and the opposite at the rural site (total female = 11, total male = 21). In order to gain some insight into my ability to detect a biologically relevant sample effect with the sample sizes that I collected, I conducted a post-hoc power analysis of the basal and the CORT stress response data sets. These analyses revealed a power of 0.473 for both basal CORT and the CORT stress response. Thus, power I observed for this study is lower than the standard power accepted for adequacy, 0.8. However, post-hoc analyses of power are not widely accepted among biologists or statisticians, particularly for non-significant results. This is because power is directly related to the *P* value of the statistical test performed. Thus, when a *P* value is not significant (as in this paper), power will necessarily be low. Indeed, in nearly all cases when a *P* value is greater than 0.05, the post-hoc power will be 0.5 or lower (Goodman and Berline 1994, Hoenig and Heisey 2001, Brosi and Biber 2009). Therefore, although the post-hoc power for this analysis is low, it is unlikely that it invalidates the results reported in this manuscript.

In addition, having fewer *C. picta* present at the rural site, and capturing more of them later in the season, could have biased the results. Intra-annual variation in CORT levels has been documented in other species and has been attributed to changes in behavior or to interactions between CORT and sex hormones such as testosterone. For example, baseline levels of CORT in female green sea turtles (*Chelonia mydas*) increase during the nesting season (Hamann et al. 2002). In another study, Selman et al. (2012) found that basal levels in the map turtle *Graptemys*

flavimaculata stayed largely the same for both females and males. However, females exhibited a dampened CORT stress response during the nesting season while males exhibited a heightened stress response during the nesting season. Furthermore, previous studies in other vertebrates and in some reptiles have shown a dampened stress response in all females during the nesting season (Astheimer et al. 1995, Breuner et al. 2003) or specific to gravid females during the same period (Anderson et al. 2014, Jessop et al. 2000).

Nevertheless, for the population and the species studied here, such a bias may be unlikely because the variable, date, did not have a significant effect on CORT. This may be that the case because all samples were collected during the nesting season. In fact, some of the last blood samples collected at my more human-impacted site came from female turtles who had just completed nesting, and gravid females were collected from my less human-impacted site until the day that collection ceased. Furthermore, *C. picta* females exhibit minimal intra-annual variation in CORT levels (Refsnider et al. 2015). This study compared CORT levels at four time periods throughout the year in four populations from across the *C. picta* range (TCRA, Washington, Iowa, and New Mexico). Baseline levels of CORT did not vary significantly for any population or time period except for immediately after emergence from hibernation (Refsnider et al. 2015). Still, it would be useful to assess the generality of my findings by including more paired urban and rural sites and by studying freshwater turtle species that are habitat specialists as opposed to generalists like *C. picta*. It would also be useful to collect heterophil to lymphocyte (H:L) ratios. After exposure to a stressor, H:L ratios remain elevated long after CORT levels have declined, suggesting that H:L ratios may be a more reliable measure of long-term stress than circulating CORT (Davis et al. 2008, Vleck et al. 2000). Nonetheless, given the abundance of studies that have successfully shown CORT to be an indicator of stress

induced by human activity, the CORT data collected here should be sufficient to determine if *C. picta* are stressed by the presence of humans or not.

One intriguing finding from this study is that male *C. picta* have significantly higher circulating CORT levels than female *C. picta*. Females may have depressed levels of CORT compared to males because the stressors associated with the nesting season, such as coming onto land to search for suitable nesting habitat (females may be depredated, or injured or killed by automobiles) are more substantive for females than they are for males. For example, some bird species have depressed CORT levels during the reproductive season because high CORT levels are associated with self-maintenance behaviors as opposed to breeding behaviors (Breuner et al. 2003, Palacios et al. 2012). It could be that female *C. picta* have depressed basal CORT and a depressed CORT stress response for this same reason. However, this explanation may be unlikely since previous research detected no intra-annual fluctuation of CORT levels in female *C. picta* (Refsnider et al. 2015). In species that exhibit depressed CORT levels due to nesting pressures, typically it is only during the nesting season itself that CORT levels are lowered, as opposed to being consistently lower (Breuner et al. 2003). Alternatively, male-specific stresses, such as searching for females and convincing them to mate, are more apt to elicit a heightened stress response in *C. picta*. Indeed, CORT has been associated with increases in locomotor activity due to breeding season activities in amphibians and other reptiles (Landys 2006). To determine the validity of this hypothesis, it would be useful to take intra-annual blood samples from male *C. picta* to determine if stress response is heightened only during the breeding season.

An additional notable finding is that *C. picta* appear to have higher levels of CORT compared to other species of turtle. For example, Gregory and Schmidt (2001) found CORT baselines of 6.16±2.31ng/mL in Kemp's ridley sea turtles, roughly one-half to one-quarter the

levels reported here. However, the *C. picta* results were consistent across two years of data collection, and previous work on female *C. picta* found similar high levels of basal CORT (Refsnider et al. 2015). Therefore, these high basal levels likely reflect actual circulating levels of CORT within wild *C. picta.* Furthermore, some other species of wild turtle have been shown to have higher levels of circulating CORT than those reported by Gregory and Schmidt. A 2012 paper by Drake et al. reported basal CORT levels of ~8ng/mL to ~9ng/mL (Drake et al. 2012). In addition, although the CORT stress response levels recorded for *C. picta* are higher than those documented in other species of turtle, many of these other studies took only a basal and a "time 30" blood sample. They therefore may not have captured the entire stress response (Cash et al. 1997; Gregory and Schmidt 2001; Selman et al. 2012).

The results of the present study indicate that *Chrysemys picta* does not experience heightened CORT levels in response to the extensive presence of humans during the nesting season. However, males exhibited an elevated stress response compared to females. Future studies in this and other systems should evaluate these findings for generality in freshwater turtles as a whole. Regardless, this study contributes basic data on glucocorticoid responses for a free-living, freshwater reptile, a group that is historically underrepresented in glucocorticoid studies. This information is essential for building a comprehensive data set that allows the testing of comparative endocrinological and physiological hypotheses across reptiles. As reptiles are the only ectothermic amniotes and occupy a key phylogenetic position within vertebrates, insight into their endocrinology also may help answer important questions about the evolution of this physiological system.

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Figures and Tables:

Table 1: Blood samples collected from *wild C. picta* for the years 2012 and 2013. Urban refers to the more human-impacted site, TCRA, and rural refers to the less human-impacted site, LMU. No blood samples were collected at LMU in 2012. Collection method, i.e. walking on land, post-nesting or basking trap (See Methods Section for details) is noted, as well as whether just a basal sample or an entire corticosterone stress response series was collected.

Population	Year	Collection Method: post-nesting/walking	Collection Method: basking trap	Basal Only	Stress Response Only	Total
Urban Female	2012	8	0	0	8	8
Urban Female	2013	30	1	23	8	31
Total Urban Female	2012 + 2013	38	1	23	16	39
Rural Female	2013	0	11	8	3	11
Urban Male	2012	3	0	0	3	3
Urban Male	2013	1	6	4	3	7
Total Urban Male	2012 + 2013	4	6	4	6	10
Rural Male	2013	0	21	13	8	21

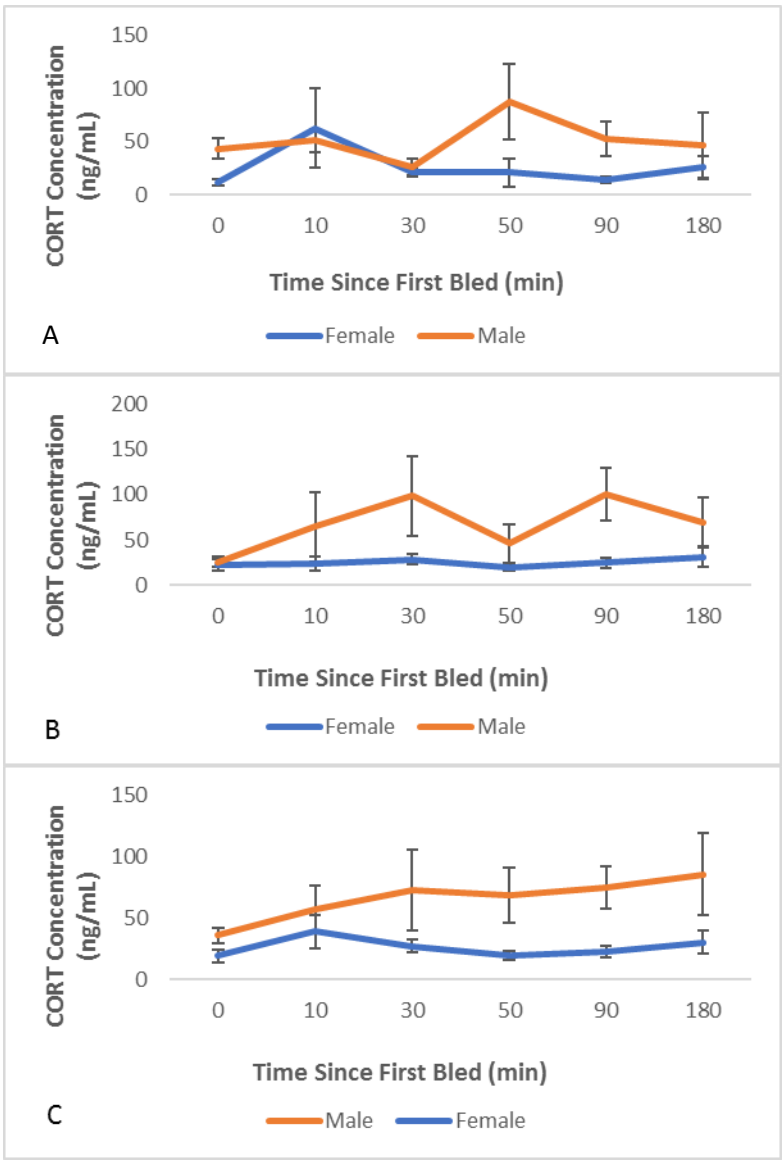

Figure 1: (A) The stress response curve for *C. picta* at LM, the less human-impacted population. Time = 0 min indicates baseline corticosterone levels (See Methods Section for details). The data are corticosterone concentration on the y-axis, and time since first bled on the x-axis. Standard error bars have been inserted for each time point for each sex. Sample sizes basal: male, n = 13, female, n = 8. Sample sizes stress response: male n = 8, female n = 3. **(B)** The stress response curve for *C. picta* at TCRA, the more human-impacted population. Sample sizes basal: male, n = 4, female, n = 23. Sample sizes stress response: male n = 6, female n = 16. **(C)** This graph represents the *C. picta* stress response curve from both the less human-impacted population, LMU, and the more human-impacted population, TCRA. Sample sizes basal only: male, n = 23, female, n = 31. Sample sizes stress response: male n = 14, female n = 19.

CHAPTER 4

EFFECTS OF AUGMENTED STRESS HORMONES ON PAINTED TURTLE DEVELOPMENT AND BEHAVIOR

This manuscript is prepared for submission to the peer-reviewed journal *Physiology and Behavior*

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Abstract – Maternal stressors can play an integral role in offspring development and ultimate behaviors in many vertebrates. Increased circulating stress hormones can be reflected in elevated concentrations in ova, thus providing a potential mechanism for maternal stress to be transmitted to offspring even in taxa without parental care. In this study, we assessed the potential impacts of augmented stress hormones on offspring development and anti-predator behaviors in a freshwater turtle, *Chrysemys picta*. We exposed *C. picta* eggs to biologically relevant amounts of the stress hormone, corticosterone, as a proxy for maternal stressors. We allowed the eggs to incubate in the field, then measured offspring phenotypes, conducted performance trials, and simulated nest emergence in a field experiment. Exogenous corticosterone reduced survivorship to hatch, but did not affect incubation duration, offspring size, overwinter survival, or size after hibernation. In performance trials, this hormone treatment reduced the frequency of righting, yet enhanced the righting speed of neonates. Regardless, these performance differences did not detectably alter survivorship in the nest emergence experiment. These results lend insight into

the potential effects of maternal stress levels on offspring phenotypes, as well as the robustness of offspring fitness to altered levels of maternal stress in freshwater turtles.

Keywords Incubation, righting response, behavior, steroids, dispersal

Introduction

The sources of phenotypic variation among individuals, and the consequences of this variation, are of fundamental interest to biologists because this variation provides the raw material on which natural selection acts [42,66]. Biologists traditionally view an individual's phenotype as resulting from its genotype, the environment, and interactions between the two [42]. However, there is growing recognition that individual phenotypes can be influenced, sometimes dramatically, by other individuals in the population. This subcategory of environmental effects has received less attention than other components of environmental effects. Nevertheless, these effects are ubiquitous and multifaceted and have important ecologically and evolutionarily relevant effects [2,42]. For instance, litter size significantly affects personality types in rats, with rats born from small and large litters most likely to exhibit fearful personality traits, including being less bold and explorative [51]. Similarly, Nazca boobies exhibit lifetime alterations in behavior based on interactions with adults when they are nestlings. Indeed, the degree to which a pre-fledged bird has been targeted for maltreatment reflects its own infliction of maltreatment to young birds later in life [43]. Perhaps a more striking example is the change in growth, and even sex, documented in clown fish in response to change in social status. The female clown fish is the largest member of the group and, if she dies, the breeding male changes sex to become the breeding female while the largest non-breeding male becomes the breeding male. Even the growth of subordinate males is maintained within well-defined size differences between the subordinate rank fish [10]. Taken together, these works (and many others) show that individual

phenotypes can be influenced, at behavioral, physiological, or morphological levels, by other individuals in a population.

Maternal effects are a prominent pillar of this perspective [42]. They occur when a mother's environment or genotype influences the phenotype of her offspring independent of the genetic information she cedes to her offspring [2]. Mothers determine when and where they will produce offspring. Additionally, the quality of the mother's environment can alter variation in her body size, condition, and physiological state at the time of reproduction. These factors can influence her transgenerational transmission of substances such as yolk amount, lipids, hormones, proteins, epigenetic factors, and xenobiotics [42,40,12,30]. These substances may directly (via maternal programming) or indirectly (via offspring sensitivity to maternally transmitted factors) influence offspring development and adult phenotype [42]. The extent to which these transgenerational factors influence offspring phenotype and fitness varies across taxa and type of effect. For instance, transgenerational effects are often adaptive and may inform offspring phenotype, including physiology, behavior, and morphology, according to conditions experienced by recent ancestors [46]. However, one growing concern is that maternal stress elevated by numerous anthropogenic factors could negatively affect offspring [54,45,34,35].

A major element that could be transmitted due to heightened maternal stress during vitellogenesis and reproduction, are glucocorticoids, with corticosterone (CORT) acting as the primary glucocorticoid. Stress hormones like glucocorticoids are involved in regulating vital behaviors such as locomotor activity and feeding, as well as metabolic processes such as lipid metabolism [32]. Heightened levels of glucocorticoids are also associated with the vertebrate stress response. In response to a stressor, circulating glucocorticoids increase in concentration in the blood stream within minutes or hours after the event. They help to keep the animal in a

heightened state of awareness after experiencing an acute stressor, and additionally promote recovery from stress and re-establish homeostasis [6,13,52,53].

Such hormones can also profoundly influence fitness of developing embryos in various vertebrates [56,24,19,16]. In particular, heightened levels of CORT in mothers can adversely affect offspring development. For example, elevated levels of maternal CORT depress offspring immune function in yellow-legged gulls [54] and reduce the reproductive success of female offspring in zebra finches [45]. In addition, elevated levels of cortisol shorten offspring length at hatching in reef fishes [34,35] and increase mortality and malformations in Atlantic salmon [19]. Similar studies of turtles have been limited, but recent research suggests that offspring phenotypes are buffered from elevated maternal stress [11]. Despite these generally adverse findings, some researchers view this mechanism of maternal transfer as an adaptation that prepares the young for the post-birth environment [33,24]. As maternal transfers of stress hormone levels have lasting impacts on offspring stress reactivity in many species, they may affect how individual offspring react to acute and chronic stressors throughout their lifetimes.

To better understand how stress hormones affect offspring phenotype and fitness, we experimentally applied CORT to painted turtle (*Chrysemys picta*) eggs in natural incubation conditions. In addition to a control, we created three treatment groups: a low CORT dosage, a medium CORT dosage, and a high CORT dosage that reflect levels of CORT quantified in yolks of slider turtles (*Trachemys scripta*) [11]. We measured phenotypic traits critical to hatchling fitness, such as length and mass, and performed righting trials (to mimic predation events) and a release experiment (to replicate dispersal in the wild). We hypothesized that high levels of CORT, consistent with extensive maternal stress, would adversely affect embryos and offspring. Specifically, we predicted that embryos from higher CORT treatments would have reduced

hatching success, take longer to hatch, and emerge from the eggs as smaller hatchlings [37,65]. Additionally, we expected hatchlings from higher CORT treatments to perform more poorly in righting trials and in dispersal abilities compared to offspring from the control and lower CORT treatments [38]. Our research provides key insights into how maternal stress hormone transfer in freshwater turtles might impact the phenotypes, performance, and fitness of offspring.

Methods

Field Site and Study Species

The painted turtle is a mid-sized freshwater species that occupies ponds and backwaters of larger rivers in North America. Adult painted turtles are primarily active from April to mid-October. Males court females in late summer and females often lay two clutches early the following summer, typically two weeks apart. These eggs incubate for 2-3 months, hatching in August and September. Intriguingly, hatchlings typically overwinter in the nest [20]. Upon emergence from the nest in spring, neonates must reach water quickly to evade terrestrial predators, avoid desiccation, and find food.

The field site where this research was conducted, Thomson Causeway Recreational Area (TCRA), is located in the backwaters of the Mississippi River in remnant sand prairie habitat in Illinois. It is a popular RV campsite and daytime recreational use area. Despite high numbers of recreation enthusiasts, *C. picta* frequently nest near humans [5,60]. This extensive human presence could potentially elevate maternal stress.

Fieldwork

From 13-21 June 2015, 20 *C. picta* clutches were retrieved after nest completion. As female *C. picta* can begin nesting as early as mid-May, these clutches were likely second clutches produced

by mothers who had already laid eggs once that summer. Nests were screened, and only those that contained at least eleven eggs (about the average clutch size) [41,55] were used for this research. Each clutch was carefully unearthed and divided into five categories: (1) a group frozen to assay baseline CORT levels in the future (one egg), (2) a control group that was not manipulated (one egg), (3) a low CORT treatment group (three eggs, 0.05ng/0.5µL), (4) a medium CORT treatment group (three eggs, 0.25ng/0.5µL), and (5) a high CORT treatment group (three eggs, 0.5ng/0.5µL) [11,65]. The limited research on CORT in reptile eggs indicates that our treatment doses are likely within a biologically relevant range for freshwater turtles [62,63,11]. Indeed, the highest treatment is considerably lower than previous reptile CORT dosing experiments [65]. In sum, then, 20 eggs were frozen, 20 comprised the control group, and 60 were in each of the three CORT treatment groups. The treatment was topically applied in a 5µL bolus [65]. As levels of endogenous steroids are highest when eggs are first oviposited [4,11], each bolus was applied within 24 hours of oviposition (usually within 2 hours).

Clutches removed from their maternal nests were placed into artificial nests in a randomized block design. Five total blocks of four nests each (20 clutches total) were constructed; the center of each nest was roughly 50 cm apart (Figure 1). Each block was constructed in a location where mothers were observed nesting in previous years. Eggs were inserted into artificial nests 24 hours after treatment, as previous research shows levels of endogenous steroids are highest when eggs are first oviposited [4,11]. Exogenously applied steroids are rapidly absorbed into the egg, including the embryo [47,48]. Thus, this method ensured that treatments were not transferred among different eggs in a nest. As one egg from each clutch was frozen, only 10 eggs were placed into each artificial nest. Before placing the eggs in their artifical nests, we weighed and uniquely labeled each egg with a felt-tip marker.

Each artificial nest was excavated to 10cm depth, which is typical for this population [40]. Additionally, each clutch was protected from predators with 1cm mesh aluminum hardware cloth secured with tent stakes.

Lab Work

In mid-August, the nearly hatched eggs were excavated and transported to Iowa State University. The eggs were weighed and placed in plastic shoeboxes filled with moist vermiculite at -150 kPa. These shoeboxes were then kept in incubators maintained at 28°C [40]. Eggs were checked twice daily for pipping (when the eggshell is slit by the eggtooth on the snout of the hatchling turtle, indicating that it is beginning to hatch). Once pipping was verified, a bottomless paper cup was placed over the egg to ensure that each hatchling could be correctly identified when it emerged from its egg. Within 12 hours of hatching, turtles were weighed to the nearest 0.01 g and their plastron lengths were measured to the nearest 0.1 mm. Once hatched, turtles were housed individually in 0.47L plastic cups and periodically misted to prevent dessication. Hatchlings were kept at 21°C and housed in a dark incubator, reflective of the subterranean nest environment experienced until the spring after their birth.

Prior to hibernation, hatchling plastrons were photographed to ensure accurate re-identification. Hatchlings were overwintered at ISU in individual 0.47L plastic cups. We began lowering the incubator temperatures on 15 October 2015, and gradually reduced the temperature to 5°C over the span of two weeks. Hatchlings were misted once a week to prevent dessication. In late-March, we gradually raised incubator temperatures to 21°C. On 20 April 2016, hatchlings were removed from hibernation, re-weighed and re-measured. Three days after the hatchlings emerged from overwintering, righting trials were performed.

Righting trials were conducted whereby a turtle was placed on its back and the time to right itself was measured. Righting trials were performed in a fully-lit room at 21°C between 1200 hour and 1600 hour to minimize any impacts induced by time of day. For each righting trial, the hatchling was placed on a 24x24 cm board covered in 423 Wetordry™. The standardized usage of this material ensured that each hatchling was tested on the same surface. Additionally, this substrate mimics a hardened sandbar and is therefore representative of conditions that hatchlings could experience in the wild. Each turtle was flipped five times with a 15 second break between each trial in order to stimulate the defensive righting response a hatching would perform if attacked by a predator [44]. For each trial, a randomly chosen turtle was removed from its cup, held ventral (plastron) side-down in the palm until it appeared calm, and then inverted on the board. Timing began when the dorsal side of the turtle (carapace) touched the board and was stopped when the turtle had righted itself onto its plastron. Time to start, time to right, overall righting time, fastest time, whether the individual righted or not, and how many times the individual righted were used in statistical analyses.

Release Experiment

On 9 May 2016, neonatal turtles were released into the center of a circular drift-fence arena constructed in the sand prairie adjacent to the TCRA. This arena had a radius of 32m, which is the average distance of *C. picta* nests from water at the TCRA [23], and 40 4.5L plastic jars buried at equal intervals along the internal perimeter of the fence to recapture dispersing turtles. Neonates were released by clutch into 20 artificially constructed depressions (10cm deep) in the center of the arena. Overall, 14 control, 46 low CORT, 39 medium CORT, and 38 high CORT turtles were used in this release experiment. The drift fence was monitored daily at 0800, 1200, 2000 hours from 9-25 May; date, time, and pit location for each captured turtle were

recorded. As most dispersing *C. picta* offspring at this site successfully reach the water within four days, any turtles not recaptured during our monitoring period were assumed dead [40,64].

Statistical Analyses

All statistical analyses were conducted using SAS software (SAS 9.4, SAS Institute Inc., Cary, NC, USA). Prior to analysis, normality of data was assessed graphically using boxplots, histograms, and q-q plots [68], and then with Shapiro-Wilk tests. Covariates were identified using a literature review and knowledge of the study species to determine which independent variables would likely have a biologically relevant effect on the dependent variable in addition to the effect of treatment group. Furthermore, we imposed contrast statements comparing all possible CORT treatment groups and assessed models for linear and quadratic effects of CORT treatment group. We did not adjust for multiple statistical tests; rather, we focused more on interpreting P-values in light of previous research on the ecology of this species and likely biological relevance.

The effects of CORT were evaluated on survivorship to hatch, incubation duration, plastron length (PL), and hatchling mass using the model:

$$\boldsymbol{Y = \mu + \text{CORT treatment group} + \text{block} + \text{initial egg mass} + \varepsilon}$$

Clutch nested within block was treated as a random effect. CORT treatment group was the treatment applied to the egg after oviposition (control, low, medium, and high CORT), block is the block in which the clutch was incubated, and initial egg mass is mass of the egg immediately post-oviposition. These were all considered potential independent variables. For survivorship to hatch, the ANOVA was conducted using the logistic regression model procedure (Proc Glimmix). For the other three traits, the ANOVA was conducted using the general linear model procedure with mixed effects (Proc Mixed).

The effects of CORT then were evaluated on overwinter survival, PL, and mass with the model:

$$Y = \mu + \text{CORT treatment group} + \text{block} + \text{pre-hibernation PL or mass} + \varepsilon$$

Clutch nested within block was again treated as a random effect. Overwintering survivorship, with pre-hibernation PL as a covariate, was analyzed using Proc Glimmix. In turn, post-hibernation PL and mass, with pre-hibernation PL and mass, respectively, as covariates, were analyzed using Proc Mixed.

The effects of CORT were analyzed to determine how long a hatchling waited before it attempted to right (time to start), the overall amount of time it took a hatchling to right (time to right), how long it took a hatchling to right once it started righting (overall time), tendency for a hatchling to right, how often a hatchling righted, and fastest righting time using the model:

$$Y = \mu + \text{CORT treatment group} + \text{block} + \text{post-hibernation PL} + \text{clutch (block)} + \varepsilon$$

Hatchling ID was treated as a random effect to force Proc Phreg to treat the righting data as repeated measures since each turtle was tested five times. Importantly, clutch nested within block was added to the model as a fixed effect because Proc Phreg only allows for one random effect and the potential impact of clutch was necessary to consider.

The effects of CORT were evaluated on offspring survival to the drift fence, location at recapture, days spent in the arena, and time of day a turtle was recaptured using the model:

$$Y = \mu + \text{CORT treatment group} + \text{block} + \text{post-hibernation PL} + \varepsilon$$

Clutch nested within block was treated as a random effect. Whether a neonate was recaptured was analyzed using Proc Glimmix; the other three variables were analyzed using Proc Mixed.

Finally, the effect of CORT was analyzed on mass at recapture using the model:

$$\boldsymbol{Y = \mu + \text{CORT treatment group} + \text{block} + \text{post-hibernation mass} + \varepsilon}$$

Clutch nested within block was treated as a random effect. Change in mass during migration was analyzed using Proc Mixed.

Results

Hatching success varied from 82-95% among the four treatment groups. CORT treatment nominally did not appear to affect survivorship to hatch ($F = 1.79$, $P = 0.15$). However, assessing the linear effect of CORT revealed that survivorship to hatch decreased as the amount of CORT applied to eggs at oviposition increased ($t = 1.93$, $P = 0.05$) (Figure 2). CORT treatment did not substantively influence incubation duration ($F = 0.96$, $P = 0.41$), hatchling PL ($F = 0.65$, $P = 0.58$), or mass at hatch ($F = 0.50$, $P = 0.68$). Instead, initial egg mass played the most important role in determining hatchling PL ($F = 118.69$, $P < 0.0001$) and mass at hatch ($F = 295.79$, $P < 0.0001$).

Overwinter mortality ranged from 16-24% among the four treatment groups. In contrast to its impact on embryos, CORT treatment did not affect overwintering survivorship ($F = 1.21$, $P = 0.31$). Pre-overwintering PL was an important predictor of overwintering survivorship ($F = 4.78$, $P = 0.03$), with larger hatchlings more likely to survive to the spring (Figure 3). The average size of a hatchling that survived overwintering was 24.43mm, whereas the average size of a hatchling that did not survive overwintering was 24.05mm. As with the hatchlings, CORT treatment did not influence post-hibernation PL ($F = 0.55$, $P = 0.65$) or mass ($F = 0.94$, $P = 0.42$). Instead, PL measured at hatch positively predicted post-hibernation PL ($F = 281.08$, $P < 0.0001$) and mass at hatch likewise positively predicted post-hibernation mass ($F = 243.13$, $P < 0.0001$).

Roughly 83% of the hatchlings righted themselves at least once in the five righting trials. CORT treatment did not affect how long a turtle took to start righting (χ^2 = 0.51, P = 0.56), to complete righting (χ^2 = 0.39, P = 0.55), overall righting time (starting time – righting time) (χ^2 = 0.49, P = 0.66), or whether the turtle chose to right (χ^2 = 0.66, P = 0.37). Nevertheless, turtles from the control treatment righted more often than hatchlings from any of the CORT treatments (χ^2 = 0.81, P = 0.04), but did so more slowly than turtles from any of the CORT treatments (χ^2 = 0.89, P = 0.03) (Figure 4).

Of the 137 neonatal turtles released into the drift fence arena, 106 were recovered in the pits in 4 d. CORT treatment did not affect the probability that a turtle was recaptured (F = 0.37, P = 0.78). Similarly, CORT treatment did not alter where turtles were recaptured along the drift fence (F = 1.61, P = 0.19). All individuals were recovered in the 15 pits closest to the nearby slough, with the highest percentage of turtles (19%) obtained from the pit roughly in the middle of this distribution. Additionally, CORT treatment did not influence mass lost while in the arena (F = 1.31, P = 0.28) or how many days the turtles spent in the arena before reaching the drift fence (F = 1.19, P = 0.32). Indeed, the only variable measured that CORT treatment may have affected was time of day that turtles were recaptured in the pits (F = 2.32, P = 0.08), reflected in a quadratic effect of CORT treatment (t = -1.95, P = 0.05). In other words, neonates from the control group were most likely to be recovered at the noon check (12:00), whereas turtles from the CORT treatment groups were most likely to be recovered at the evening check (20:00) (Figure 5). Only three turtles were recovered at the morning check (8:00), so they were excluded from this analysis.

Discussion

This study sought to simulate the transgenerational transmission of maternal hormones to egg yolk to elucidate the impacts on embryos and offspring reared in the wild. Painted turtle embryos treated at oviposition with differing levels of CORT, as a proxy for maternal stress, displayed a linear effect on survivorship to hatch. That is, as CORT dosage increased from none (the control) to high, probability of surviving to hatch decreased. Further, CORT treatment reduced the frequency of righting ability, yet enhanced the righting speed of neonates. Although intriguing, these performance differences did not translate to altered survivorship in the release experiment. It is possible that if CORT had not had such a strong effect on survivorship to hatch, effectively eliminating almost 20% of hatchlings from the high CORT treatment and around 17% of the hatchlings from the medium CORT treatment, we would have seen more deleterious effects of CORT on hatchling phenotypes. Additionally, size of hatchlings significantly affected overwintering survivorship. This differential survivorship potentially reduced the likelihood that our statistical measures would be able to pick up CORT treatment effects on subsequent righting and dispersal ability.

Perhaps our most significant finding was a linear trend in survivorship to hatch based on CORT dosage. Embryos exposed to the medium and high CORT dosages were significantly less likely to hatch than embryos exposed to the low CORT dosage or the control group. Most embryos that did not hatch either failed to develop entirely or were stunted. This outcome is in accordance with previous work on birds and reptiles [25,52]. Although a prior study has addressed the simulated effect of elevated CORT levels in eggs on offspring development in slider turtles [11], our work differs in key ways. Eggs in our study incubated in the field, rather than in the laboratory [11] and thus experienced natural temperature fluctuations. This

methodological difference might explain why we detected an effect of CORT dosage on survivorship to hatch, a fitness trait unaffected by CORT in the earlier study [11]. Still, as previous research has suggested [56,24,16,11], the effects of transgenerational maternal stress are nuanced and multifaceted. Thus, comprehensive studies on numerous different species are needed to identify generalities regarding the roles of transgenerational maternal stress.

The other notable results of our work -- how many times a hatchling righted in performance trials, how quickly it righted during performance trials, and time of day recovered from the circular drift fence arena -- have unclear biological significance. The righting response in turtles is often considered a proxy for fitness [21,58], and involves placing a turtle on its carapace and measuring how long it takes to right itself, among other measures, such as latency to right [1,17,11]. None of the qualities of righting performance that we measured correlated with changes in dispersal survivorship among treatment groups. Similarly, Carter et al. 2016 measured how many times hatchlings righted during performance trials as well as how quickly they righted. They also detected no correlation between righting behavior and dispersal success [11]. These results contrast with previous studies that assumed the righting response was a trait critical to hatchling fitness, as hatchlings that are slow to right themselves should be more vulnerable to predation, desiccation, or other threats during dispersal [8,59,22]. However, some previous research suggests that laboratory righting trials do not predict dispersal ability of neonatal turtles in the field, and thus may not be strong proxies for defense against predation attempts [39]. Furthermore, individual righting times vary substantially across trials, and thus cannot be considered strong estimates of individual fitness [17]. Yet, some research interprets righting trial performance as a general index of physical fitness rather than as a trait that is directly relevant to survival during dispersal. That is, since righting performance measures

coordination, strength, and potentially neuromuscular development, it can be considered an indirect correlate of fitness that may be directly relevant to other aspects of hatchling life than just dispersal [9,14]. For example, both how long it took hatchling turtles to right (latency to right) and time to right were positively correlated with juvenile growth rate [18], which could be a strong indicator of long-term survivorship in aquatic turtles [7]. Therefore, despite lack of correlation between righting responses and survival in the drift fence arena, righting trials may provide insight into juvenile growth rate, and thus, overall offspring fitness [18].

Additionally, our result that CORT treated hatchlings were more likely to be recovered at a different time of day than most control hatchlings could be interpreted in multiple ways. As hatchlings from CORT-treated eggs were more likely to be caught during the evening check as opposed to the noon check, they may have been waiting until later in the afternoon to begin traveling towards water. Alternatively, these hatchlings may have been slower than hatchlings from control eggs, but not enough to substantially affect time spent in the drift fence arena. We believe the former explanation is more likely, but this hypothesis could be tested by affixing tracking devices to monitor hatchling movement patterns. It is possible that level of circulating CORT in the hatchlings, which would presumably vary based on CORT treatment group, had differential effects on thermoregulation in the hatchlings. Thus, hatchlings from CORT-treated groups may have moved later in the day due to an interaction with elevated levels of circulating CORT and the air temperature. However, a previous study in this species did not find a correlation between air and body temperature and circulating CORT in adults [49]. Therefore, this hypothesis may be unlikely.

Regardless, these differences in time of recapture have the potential to be adaptive. For example, by traveling primarily in the late afternoon or early evening, hatchlings from CORT-

treated eggs may be more likely to avoid succumbing to common avian predators, which probably are the most prevalent group of predators on terrestrial hatchling turtles [27]. At our field site, the primary avian predators are red-winged blackbirds and robins. American robins forage for invertebrates and small vertebrate prey in the early morning and late afternoon [26], preferring plant-based food later in the day [67]. Similarly, during the spring red-winged blackbirds leave their roosts around dawn, and do not return until around sunset. However, their return times vary by weather, with birds returning later in the day on sunny days as opposed to rainy days [36]. Thus, depending on the prevalence of different avian predators (and even weather patterns), time of day that hatchling turtles traverse the landscape to find water may be adaptively significant. For example, if red-winged blackbirds were the primary predator at a field site that experienced frequent rain events, turtle hatchlings from eggs with high CORT levels may have a selective advantage because they would likely travel later in the day after the birds had returned to their roosts.

It may be useful to replicate this experiment in locations with higher predation pressure. Over 77% of offspring were recaptured in our study, which is comparable to recapture rates found in other release experiments [11,40,64,15,61]. For example, Mitchell et al. 2013 experienced recovery rates of 77% at TCRA as well [40], and Carter et al. 2016 had recovery rates of 89% at their Banner Marsh field site [11]. However, it may be that these experiments were conducted at sites that have relatively low hatchling predation, or during years where predation was low, perhaps due to decreased predator abundance. Other release experiments have reported substantially lower recapture rates. For example, Janzen et al. 2000 reported recapture rates of just 34% at their field site at Stump Lake near the eastern edge of the Illinois River [28]. Thus, some field sites do appear to suffer from higher rates of predation than others.

Furthermore, in a later paper, hatchling survivorship was counted at 41% at the same field site [29]. In addition, at the field site used in this paper on the Mississippi River, Kolbe and Janzen reported recaptures rates of 62.9% in 2001 [31]. Thus, even within the same field cite predation rates on hatchlings may vary by year. Perhaps CORT treatment group would contribute to differential survivorship during terrestrial migration of hatchlings in environments with heightened predation, in years of heightened predator density, or in those with novel predators.

This area of research has many other potential future directions. For instance, CORT treatment reduced survivorship to hatch even though external ambient conditions were moderate. Specifically, average air temperature in July 2016 was 23.0°C (data obtained from Clinton, IA, USA; National Climatic Data Center (www.ncdc.noaa.gov)), compared to a long-term average of 23.9°C [55]. It may therefore be intriguing to replicate this aspect of the study in a more severe (be it extreme heat or drought) summer more reflective of expected future climate conditions. Perhaps the effect of CORT treatment on survivorship to hatch would be amplified under more adverse incubation conditions. Alternatively, CORT treatment could improve embryonic hatching success under such stressful abiotic developmental conditions [25,33]. In addition, it may be insightful to repeat this study but apply CORT directly to the mother during vitellogenesis rather than to the eggs after they are laid [30,50]. For example, Robert et al. 2009 applied CORT transdermally to pregnant garter snakes so that the embryos would be exposed to CORT as they developed [50]. However, garter snakes are viviparous and turtles are oviparous. Thus, development of turtle embryos occurs in the eggs after they are laid, so our methodology almost certainly captured the nature of exposure to maternal stress hormones during offspring development. Furthermore, Janzen et al. 2002 manipulated dihydrotestosterone, estradiol-17β, and testosterone in slider turtles to determine if circulating levels would be reflected in yolk

levels of eggs. Compared to control females, females treated with hormones (via silastic implants) not only exhibited increased circulating steroid levels, but also had a six-fold increase in testosterone concentrations in egg yolk [30]. This research supports the contention that hormone concentrations in yolk reflect concentrations of circulating hormones in the bloodstream [30]. It also supports the view that topical application of CORT to recently laid eggs serves as a proxy for circulating levels of maternal CORT.

Conclusions

In sum, elevated CORT levels during offspring development reduced survivorship to hatch. In addition, CORT treatment reduced the frequency of righting during performance trials, yet enhanced the righting speed of neonates. These performance differences did not influence survivorship in the release experiment. The fact that CORT reduced embryonic survivorship nevertheless might lend insight into how turtle populations will fare in the future. Climate change scenarios forecast increased frequencies of extreme events [57], and turtles globally already suffer substantially from reduced availability of viable habitat [3]. Such pressures, among others, may increase maternal stress, particularly in species with low aptitude for adapting or acclimating readily to human-modified landscapes. Thus, transgenerational effects of elevated maternal stress may play an important role in population viability of turtle species into the future.

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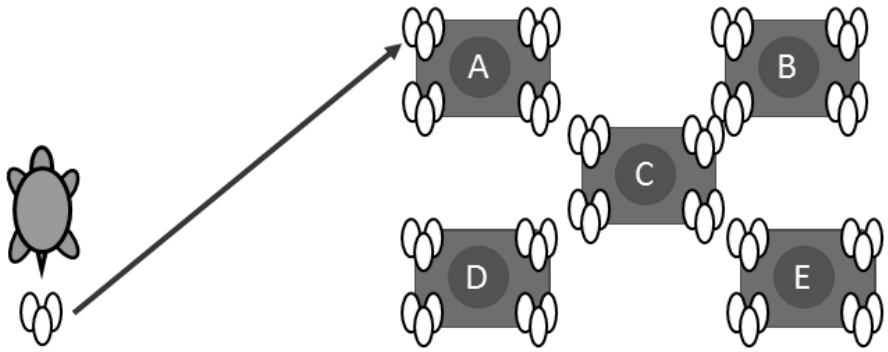

Figure 1. The randomized block design of the field incubation portion of the experiment. In total, 20 clutches of 10 eggs (at least one egg and any additional eggs beyond 11 were frozen) were randomly assigned to a block within a randomized block design. Five total blocks housing 4 clutches each were spread throughout our field site. Each block was located at a spot were females had been observed nesting in previous years.

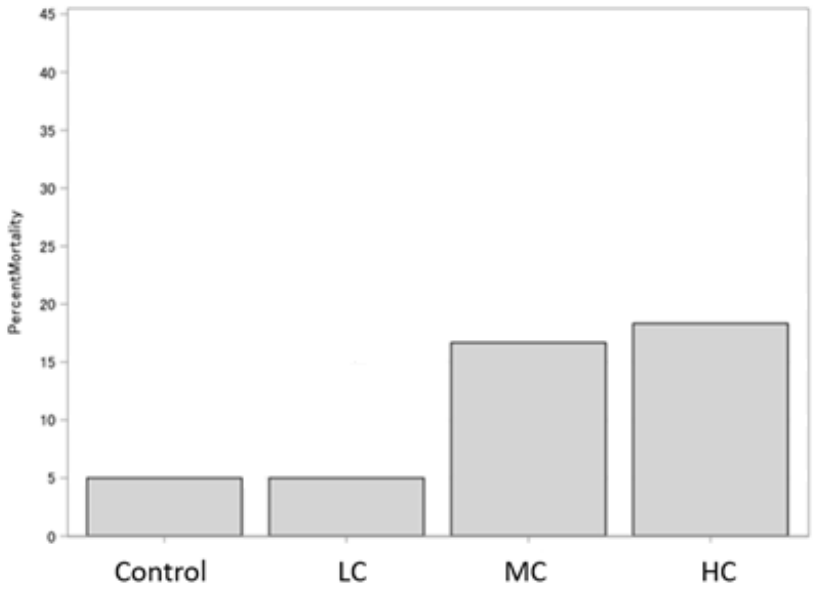

Figure 2. Percent mortality at hatch from each treatment group. The data are percent mortality (did not successfully hatch) on the y-axis and treatment group on the x-axis. Sample sizes at start: control group n = 20 eggs, low CORT (LC) n = 60, medium CORT (MC) n = 60, high CORT (HC) n = 60. Samples sizes at hatch: control group n = 19 animals, low CORT (LC) n = 57, medium CORT (MC) n = 50, high CORT (HC) n = 49.

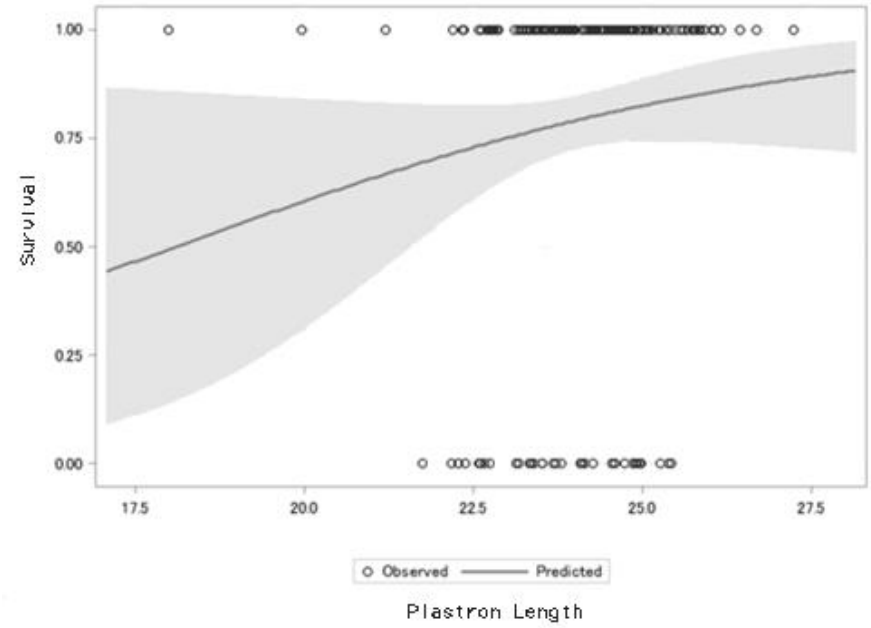

Figure 3. Probability of survival for hatchling *Chrysemys picta* during overwintering as a function of plastron length. The shaded portion of the graph represents standard errors. Open circles along the top and bottom axes represent individual hatchling turtles.

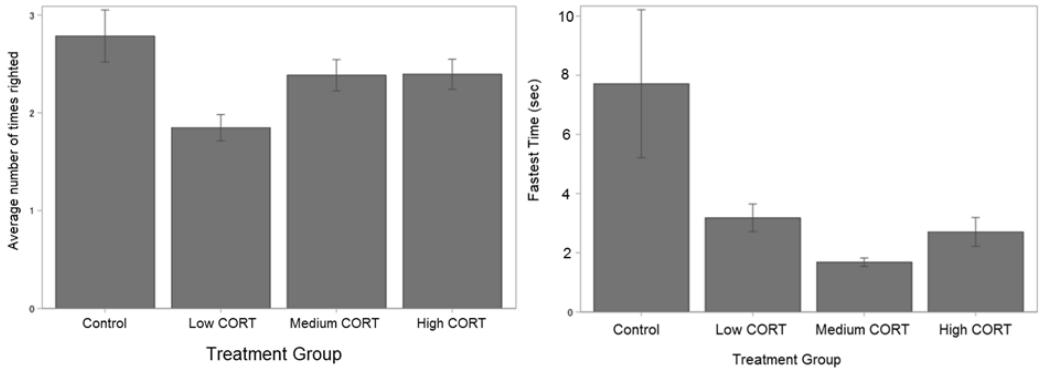

Figure 4. (A) The mean of times each hatchling righted itself by treatment group. The data are mean number of times righted on the y-axis and treatment group on the x-axis. Standard error bars have been inserted for each treatment group. Each individual was tested five times. (B) The fastest time each hatchling righted in seconds by treatment group. The data are fastest time a hatchling righted on the y-axis and treatment group on the x-axis. Standard error bars are depicted for each treatment group. Sample sizes for fastest time righted: control group n = 14 animals, low CORT (LC) n = 46, medium CORT (MC) n = 39, high CORT (HC) n = 38.

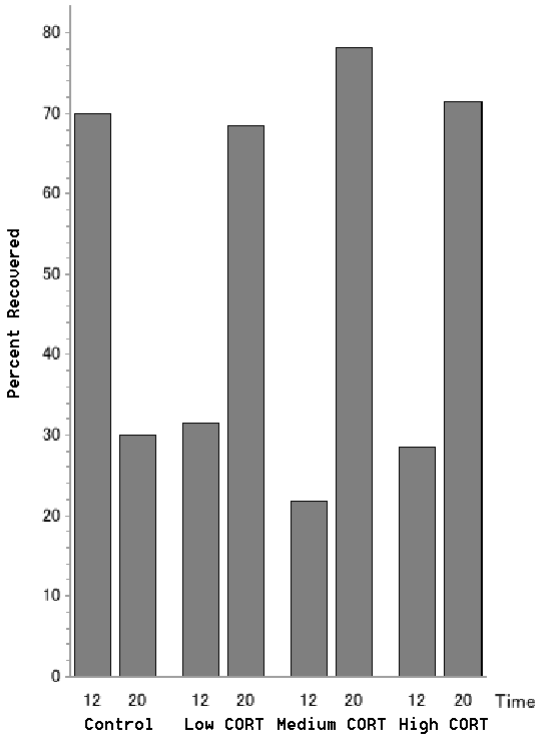

Figure 5. Time of day recovered from the circular drift fence arena per treatment group. The data are percent recovered from the circular drift fence on the y-axis and treatment group on the x-axis. The treatment groups are split by time of day recovered: 12:00 on the left and 20:00 on the right. Sample sizes at time 12:00: control group *n* = 7 animals, low CORT (LC) *n* = 11, medium CORT (MC) *n* =7, high CORT (HC) *n* =8. Samples sizes at time 20:00: control group *n* = 3 animals, low CORT (LC) *n* = 24, medium CORT (MC) *n* = 25, high CORT (HC) *n* = 20.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The goal of the studies described in this dissertation is to illustrate how freshwater turtles may respond to anthropogenic stressors, including human recreational activities, and the consequences of elevated maternal stress on offspring phenotype. Compared to other vertebrates such as birds and mammals, not many studies have documented the effects of human disturbances on behavior and physiology in wild freshwater turtles. Previous research has found detrimental effects of human recreational activity on nesting and basking activity (Moore and Seigel 2006, Selman et al. 2013), as well as buffered effects of elevated maternal stress in red-eared slider turtles (Carter et al. 2016). I augmented this meager literature through my dissertation work, examining the heretofore unmeasured circulating corticosterone levels, flight initiation distance, and the effects of elevated maternal stress levels in the painted turtle. I recorded data across multiple levels of biological organization, including measures of multiple traits within individuals (morphological, physiological, and behavioral), repeated measures over time, consequences of circulating stress hormones on reproductive output, and comparisons of populations to determine whether painted turtles can adapt or acclimate to heightened exposure to human recreational activity. This research highlights compelling findings to consider when determining how freshwater turtles are likely to respond to rapidly changing environments. It provides insight that, combined with other lines of research -- such as whether turtles with temperature-dependent sex determination will be able to adapt to predicted changes in atmospheric temperature (Schwanz and Janzen 2008, Mitchell and Janzen 2010, Refsnider et al. 2013), whether populations in urban environments will exhibit growth (Rhoe et al. 2011, Spinks

et al. 2003), as well as how turtles will respond to pressures from the pet trade (Schlaepfer et al. 2005, Gong et al. 2009) and habitat loss (McClenachan et al. 2006, Browne and Hecnar 2007, Quesnelle et al. 2013) -- should help us understand how these animals will respond to rapidly changing environments.

First, adult male and female painted turtles inhabiting environments that are utilized by humans for recreational activities, such as bike riding, fishing, and camping, do not exhibit altered circulating CORT levels. This means that, potentially, these turtles will not abandon quality nesting or basking sites despite exposure to high numbers of human recreational enthusiasts. Indeed, at the TCRA population, we do not see altered nesting behavior in response to intense human recreational activity (Bowen and Janzen 2008). Second, adult painted turtles modulate their flight initiation distance behavior in response to humans based on whether they are likely to encounter humans frequently in their environment. That is, turtles from populations with little to no experience with humans do not allow humans to get as close to them before fleeing as do turtles from populations that encounter humans frequently. Third, painted turtle hatchlings appear to be largely resistant to maternal stress transmitted in ovo. Still, with increased levels of stress hormone exposure at oviposition, survivorship of embryos to hatch declined. Additionally, there were some behavioral effects of heightened maternal stress in the righting trials. Nevertheless, these behavioral differences did not translate to differential success in the release experiment, indicating perhaps that the most likely time that effects of elevated maternal stress will influence offspring is during incubation.

These results point to some exciting potential future directions. The research performed for my first chapter indicates that painted turtles in highly human-impacted populations do not exhibit altered stress levels. However, the populations investigated in that study were either

minimally accustomed to the presence of humans, or were members of a population that has been exposed to high numbers of human recreationalists for generations. Therefore, it may be compelling to replicate this study in populations that have never been exposed to human recreational activity, populations that have only recently been exposed to human recreational enthusiasts, as well as populations that have been exposed to human recreational activities for several generations. Furthermore, the work from my second chapter indicates that painted turtles appear able to modify their behavior such that they no longer treat humans as potential predators in populations that are exposed to humans. However, is this true for all freshwater turtles? It would be exciting to replicate this study with other species, including the common map turtle or perhaps Blanding's turtle, a species declining throughout most of its historic range. These studies may lend insight to how generalizable my findings are across freshwater turtles. Specifically, is there a generalizable trend between common species, such as painted turtles, and rare species, such as Blanding's turtles? Finally, for my last chapter, considering that CORT treatment reduced survivorship to hatch during a mild summer, it would be intriguing to replicate this aspect of the study in a more severe (be it extreme heat or drought) summer. It is possible that an even stronger effect of CORT treatment would be detected on survivorship to hatch, as the negative effects we found may be amplified in poor incubation conditions.

These questions can be addressed using painted turtles, or other species found in and around the Thomson Causeway Recreation Area, as model organisms. Such work would be well facilitated by the already substantial knowledge of the evolutionary and ecological backgrounds of these numerous species at that field site. I hope to continue to ask such crucial and intriguing questions as I continue my career as a biologist.

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